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The photosynthetic ability of Rosa in vitro

Langford, Penny

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The Photosynthetic Ability of *Rosa in vitro*.

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P. J. Langford

P.J. Langford

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

For Grandad

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The commercial production of miniature roses a2
at Neo Plants Limited, Freckleton, Lancs.,
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ABSTRACT

There are relatively few reports in the literature concerning the photosynthetic status of shoots *in vitro*, and little assessment of the factors associated with its regulation. This thesis reports the influence of sucrose concentrations, the use of alternative sugars, the role of media osmotic potentials and various light levels on the photosynthetic and growth characteristics of rose shoot cultures, cvs. Iceberg and Peace, *in vitro*. The influence of the culture medium, more specifically the sucrose concentration, used prior to transfer to soil is also studied, to observe the effect on subsequent *in vivo* rooting, establishment and continued growth of rose shoots cvs. Peace and Fragrant Cloud.

The use of reduced concentrations of media sucrose led to a significant increase in the photosynthetic rate, although it did not prove possible to obtain sucrose-independent growth (photoautotrophism) under the culture conditions used, showing shoots to retain a net negative carbon balance. The rate of shoot proliferation could be increased by the use of glucose as carbon source, instead of sucrose, indicating the inclusion of alternative sugars to be of potential use for improved shoot culture.

The use of a pre-transfer culture medium, lacking plant growth regulators, but with the inclusion of activated charcoal, greatly improved the overall size and vigour of rose shoots. Shoots were successfully rooted

in vivo, although the use of varying levels of sucrose in the pre-transfer medium had no clear influence on the subsequent rooting and establishment of shoots. Thus establishment would appear to depend primarily upon the development of leaves initiated *in vivo*, the latter possessing increased ('normal') photosynthetic rates, chlorophyll contents and, perhaps more importantly, a positive carbon balance with a net uptake of carbon. The *in vitro* leaves may however, make some contribution to the growth of the establishing shoot, especially if, as in this study, they can be induced to develop some degree of photosynthetic competence following transfer.

Abbreviations

ABA	abscissic acid
BAP	6-benzyl-aminopurine
CO_2	carbon dioxide
cv	cultivar
FW	fresh weight (g)
GA_3	gibberellic acid
IAA	indoleacetic acid
IRGA	infra-red gas analysis/analyser
LA	leaf area (cm^2)
MS	Murashige and Skoog's (1962) mineral salts
N_2	nitrogen
NAA	naphthaleneacetic acid
O_2	oxygen
OP	osmotic potential
RH	relative humidity

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of rose cv. Fragrant Cloud prior to and after transplanting to soil, following shoot elongation on medium containing 10, 20 or 30 gl^{-1} sucrose.

CHAPTER 1.

INTRODUCTION

The micropropagation of plants *in vitro* is a complex process involving a series of defined stages, the exact number of which often varying between species and laboratories depending upon the degree of sub-division required to optimalise the developmental process eg. shoot proliferation, rooting etc. The following summary is taken from three main reports which present an overall discussion of the micropropagation process (Murashige, 1974; Debergh and Maene, 1981; Conner and Thomas, 1982).

Stage 0. The preparation of stock material prior to explant removal, involving growth under clean, hygienic conditions. This is not often recognised as a separate stage, although may clearly enhance explant growth and quality in culture, reducing the possibility of infection and enabling more rapid establishment in culture. A healthy stock plant will give rise to healthy explants.

Stage 1. The establishment of aseptic cultures *in vitro*. After removal from the mother plant, explants are surface sterilised (commonly with sodium hypochlorite).

Stage 2. The multiplication of propagules in culture, involving the maintenance and increase of stock.

Stage 3. The preparation of propagules for transfer to soil. This may include an elongation period, during which time shoots are induced to increase in size and, if possible, quality. This is followed by the induction of roots *in vitro*, or, if rooting *in vivo*, by direct transfer of shoots to soil.

Stage 4. The transfer of propagules to soil ie. weaning and acclimatisation. This stage necessitates the provision of high humidity for the first few weeks, so as to enable cultured shoots to acclimatise to the *in vivo* environment. This period must obviously therefore, provide for optimal establishment so that the care taken during the *in vitro* stages is not wasted and large numbers of plants are lost.

The work presented in this thesis involves a study of the photosynthetic characteristics of rose shoots *in vitro*, with a view to improving the weaning and acclimatisation of plantlets on transfer to soil. Whilst there are numerous reports in the literature discussing the photosynthetic abilities of single cell and callus cultures, there are very few involving whole shoots. The potential use of such studies are of undoubted commercial interest, and the whole area merits much greater attention. This thesis may make some inroads into what is a complex and, at present, neglected area of plant tissue culture.

1.1 The Problems of Weaning and Acclimatisation

There are two main problems associated with the transfer of tissue-cultured plantlets from the *in vitro* to the *in vivo* environment, apart from those concerning factors of disease susceptibility. The first is in their inability to control water loss, which leads to very rapid wilting and desiccation of plant material when

taken out of culture. During the weaning process therefore, humidity levels have to be maintained very high for the first few days, so preventing excessive water loss and allowing plantlets to acclimatise to the new conditions. This is achieved through the use of misting or fogging units, humidity tents (Loach, 1979; Grange and Loach, 1984) and the application of foliar antitranspirants (Wardle et al, 1979), although there are reports of such foliar sprays being rather ineffective and in some cases even stunting plantlet growth (Sutter and Hutzell, 1984).

The second problem is that cultured plantlets are photoheterotrophic, with low levels of photosynthetic activity in vitro ie. they are relying on the exogenous supply of sugar (usually sucrose at 2 or 3%) provided in the culture medium for their growth. When transplanted to soil, they have to develop photosynthetically competent new foliage, which results in an inevitable delay in the resumption of new growth. Both these problems and the whole area of establishing plantlets from tissue-culture is reviewed by Conner and Thomas (1982) and Dunstan and Turner (1984).

1.1.1 The humidity factor

Humidity levels within the environment of the culture vessel are very high, approaching 100% relative humidity (RH), and it is this factor which affects both the morphology and anatomy of plant material in vitro. Leaves

of cultured plantlets are characterised by reduced levels of epicuticular waxes, abnormal stomatal functioning and a reduced stomatal frequency, all of which contribute to the high rates of water loss and poor control of cuticular transpiration (Grout and Aston, 1977, 1978a; Sutter, 1982; Conner and Conner, 1984). These cultured leaves also lack a distinct palisade mesophyll and have a greater percentage of air space within the spongy mesophyll (Brainerd et al, 1981; Wetzstein and Sommer, 1982, 1983), ie. the leaves have a reduced dry matter content compared with greenhouse-grown plants.

The reduced levels of epicuticular waxes result in a much reduced waterproofing of the plantlet leaf so that cuticular transpiration cannot be minimised (Grout, 1975; Sutter and Langhans, 1979). The inability to control water loss however, is more immediately related to the lack of stomatal closure seen either on excision of cultured leaves or on their treatment with ABA, high CO_2, mannitol, darkness etc. In greenhouse leaves of Malus, ~ 96% of stomates closed in response to these treatments, whereas less than 5% of stomata of in vitro leaves were induced to close (Brainerd and Fuchigami, 1982). Further work with Chrysanthemum, leads to the suggestion that the guard cells are capable of both inflation and deflation, and that the biochemical events of guard cell movement are unimpaired, but that the pattern of microfibrils is such that the stomata in the in vitro environment are kept open (Wardle and Short, 1983).

The process of acclimatisation involves the weaning of plantlets from the high levels of humidity found in culture to those of the greenhouse environment (Brainerd and Fuchigami, 1981). During this process there may be limited changes in the stomatal response and wax deposition of cultured leaves, although adaptation depends primarily on the development of new leaves as opposed to the growth of those already present (Donnelly and Vidaver, 1984b; Fabbri et al, 1986). Indeed, there are several suggestions that the leaves persistent from culture do not grow following transfer to soil and simply act as storage organs (or 'cotyledonary structures'), providing the developing leaves with nutrients accumulated during their time in culture (Wardle et al, 1983a; Short et al, 1984; Grout and Millam, 1985; Short et al, 1985).

There are several studies following the anatomical and morphological development of plantlet foliage after transfer to soil (Donnelly and Vidaver, 1984a; Donnelly et al, 1985). The persistent foliage of Rubus idaeus L. shows little or no change over time, with only a minimal amount of growth. Over successive weeks following transplanting, leaves are developed which show a graded anatomy and appearance between those of the cultured and greenhouse-grown material. This presumably reflects the growth of new foliage initiated in vivo, away from the 'adverse' conditions of the in vitro environment.

Studies with seedlings of Brassica show that a

decrease in humidity leads to the deposition of large amounts of surface wax (Baker, 1974). Similar studies with shoot cultures of Chrysanthemum and Brassica, cultured under a range of RH (15-100%) in vitro, also show that reduced levels of RH induce increased wax deposition (Sutter and Langhans, 1982). However, growth is severely retarded at 15% and 30% RH, with a very high mortality rate (70%). Optimum growth rates, similar to those at 100% RH, are seen for plantlets grown at 80% RH. Such plantlets also show normal stomatal physiology and water relations and can be transferred to soil without humidity protection and wilting does not occur (Wardle et al, 1983b; Short et al, 1985; Short and Roberts, 1986).

Thus it may be possible to wean tissue-cultured plantlets within the in vitro environment itself, so removing the need to provide a means of humidity protection and set aside large areas for weaning etc. This could significantly reduce both the cost and length of the acclimatisation and establishment period, although the possibility that they may spend longer in the growth room, and will need some method of RH control, may present practical problems, and must therefore be considered.

1.1.2 The photosynthetic problem

The study of photosynthesis and the photoautotrophic culture of plantlets ie. growth without a source of

organic carbon *in vitro*, is less well documented, yet is of importance and potential practical use within the tissue-culture system.

If photosynthetically competent foliage could be developed under *in vitro* conditions, which persisted on transplanting to soil, then the development of new foliage and establishment of plantlets would be greatly improved. The importance of photoautotrophic growth for single cells and callus could be directed towards a more physiological role, in the study of photosynthetic and respiratory metabolism, secondary metabolite production (Hagimori *et al*, 1984) and as the basic research material in, for example, mutant selection, herbicide screening, CO_2/O_2 recycling etc. (Horn and Dalton, 1984). There are numerous reports in the literature concerning the photoautotrophic growth of cell and callus cultures and these are extensively reviewed by Yamada *et al* (1978, 1982) and Chaumont and Gudin (1985).

Efforts to induce photoautotrophism simply by the omission of sucrose from the culture medium have been unsuccessful in all reported cases. It has been found necessary to increase the CO_2 concentration and light intensity within the culture environment, in conjunction with a reduction or omission of sugars from the culture medium (LaRosa *et al*, 1984; Chaumont and Gudin, 1985; Tyler *et al*, 1986). The majority of such photoautotrophic cell cultures however, show reduced growth rates, with two notable exceptions, photoautotrophic suspension

cultures of Asparagus officinalis L. and soybean both being found to give increased rates of growth (Peel, 1982; Horn et al, 1983).

The photosynthetic capacity of cultured plantlets is much lower than those of greenhouse-grown controls (Grout and Aston, 1978b; Smith et al, 1986), although shoot cultures of Bramley apple are reported to have an active photosynthetic mechanism when grown on a sucrose medium (Abbott and Belcher, 1982). Studies with Brassica meristem cultures indicate that whilst the chloroplasts exhibit light-stimulated electron transport comparable to control plants, the levels of RuBP carboxylase activity are reduced (as are chlorophyll contents) resulting in low rates of carbon assimilation (Grout and Aston, 1978b; Grout and Donkin, 1985). Such plantlets are unlikely therefore to be able to survive on a sucrose-free medium, as they have a net negative carbon balance in vitro (Grout and Crisp, 1977).

As with wax and stomatal features, the photosynthetic system in cultured leaves does not develop following transfer to soil. These leaves rapidly deteriorate, contributing very little to the carbon uptake of the establishing plantlet (Donnelly and Vidaver, 1984b; Grout and Donkin, 1985). During the acclimatisation of plantlets to soil, the first formed leaves are again transitional in their photosynthetic capicity, this being seen to increase as leaf anatomy undergoes the transition from the in vitro to the in vivo condition (Grout and

Millam, 1985). This process may be further enhanced by transplanting to higher light intensities (Donnelly et al, 1984).

Plantlets may be screened for photosynthetic competence by transferring them to sucrose-free medium. The majority of species cannot survive without an organic carbon source, indicating a net negative carbon balance. However, plantlets of Dieffenbachia, Chrysanthemum and Brassica are reported to be capable of sustaining a slow rate of growth, and still remain chlorophyllous on a sucrose-free medium, indicating a degree of photosynthetic competence (Short et al, 1984; Grout and Donkin, 1985). Indeed, oxygen evolution from such shoot cultures of Brassica and Chrysanthemum is not significantly less than that from greenhouse-grown seedlings (Short et al, 1985). Such foliage does not degenerate on transfer to soil, but persists and contributes significantly to the subsequent growth and development of the establishing plantlet.

Thus the acclimatisation of tissue-cultured plantlets requires the production of new leaves initiated within the in vivo environment, which are both photosynthetically competent and able to control water loss. From the literature, it is clearly possible to develop plantlets with normal wax and stomatal characteristics in vitro and which correspondingly do not need humidity protection during weaning. The technique of growing plantlets in vitro at reduced levels of humidity

would seem to be easy and cheap to implement, and is also likely to be applicable to the majority, if not all, micropropagated species.

The production of photosynthetically competent foliage *in vitro* is not so easily achieved. Although there are reports of a few species being able to grow in culture, albeit slowly, without an added source of organic carbon, these are by far the exception, most species surviving for less than 8–10 weeks on a sugar-free medium.

There are numerous factors of the *in vitro* environment, other than humidity and carbon source, which may be altered in order to try and optimalise the transfer of cultured shoots to soil. Light quality (Norton and Norton, 1986) and quantity (Lee *et al*, 1985) both affect plantlet growth and physiology. The results presented in this thesis, therefore, report the effects of carbon source, both type and concentration, and light quantity on the photosynthetic ability and growth characteristics of rose shoot cultures. These factors are studied with a view to conditioning shoots prior to their transfer to soil. In addition to this, any reduction in cost and labour at the commercial level must also be considered an important benefit of improved plantlet establishment (Donnan *et al*, 1978).

1.2 The Micropropagation of Rose

Rosa was chosen as the ideal plant to work with as it is widely micropropagated commercially, generally growing well in culture with fairly large, easy to handle shoots in vitro, and a good rate of shoot multiplication (Alekhno and Vysotsky, 1986).

The two cultivars chosen for this study are both popular and widely grown in gardens in this country. 'Iceberg' is a pure white Floribunda (or cluster-flowered) rose, often flushed with pink in the autumn, with a strong fragrance. It is extremely floriferous, flowering throughout the season, and shows strong, vigorous growth. 'Peace' is a Hybrid-Tea (or large-flowered) rose. It produces lightly-scented golden yellow blooms (40-45 petals) edged with pink, often up to 15 cm across. Each bloom grows singly on a very strong stem. The foliage is characteristically large and glossy, with dark green leathery leaves.

1.2.1 Commercial production

The rose is one of the ten species most commonly produced by micropropagation. Hybrid-Teas, Floribundas. miniatures, climbers, ground cover, shrub roses etc. all grow well in culture, and there are a wide range of varieties and colours commercially available (see Appendix a1, a2). Whilst some species and cultivars are available as standard lines, others are only produced under contract, which requires at least 18 months lead

time prior to delivery. It is thus very difficult to obtain precise figures for the production of micropropagated roses, as both numbers and prices fluctuate according to demand and availability. The data shown in Table 1 was obtained by writing to the companies concerned, only those producing roses on a large scale being able to provide numbers and prices etc.

In 1985 there was an apparent glut of roses, with the inevitable effect on prices (Dixon, 1986 - private communication). Royalties may also have to be paid for particular cultivars, adding anything from 4-12 pence to the price of each plant. The commercial product is a rooted, weaned plantlet, sold ready for immediate potting on under normal glasshouse conditions.

1.2.2 General culture procedures

Numerous factors are reported to influence the propagation of rose *in vitro*. The position of the node from which the axillary buds are isolated may have an effect on their growth and development in culture, although the literature is somewhat contradictory (Davies, 1980; Bressan *et al*, 1982). The culture environment itself also has an obvious effect, with factors such as light, temperature, plant growth regulators etc. all having a strong influence on shoot growth (Bressan *et al*, 1982). Culturing shoots at high light levels often results in a high degree of leaf senescence and an irradiance of 1000lux is commonly

Table 1. The commercial production of rose through micropropagation, Sept.1986 (HT=Hybrid-Tea, F=Floribunda).

Company	Types and cvs. produced, approx. nos. per annum, price per plant
Microprop Roses, LEICS.	HT (29cvs.), F (27 cvs.), miniatures (22 cvs.), climbers/ramblers (13 cvs.) ground cover/shrub (3 cvs.) A few hundred thousand in total
Munton and Fison, SUFFOLK	HT, miniatures eg. 'Rosaminis' and other contract varieties HT - 10,000, miniatures - 80,000
Neoplants Ltd., LANCS.	HT, F, miniatures (12 cvs.), climbers, ground cover and shrub (eg. 'Canary Bird' 'Snowcarpet', 'Penelope' etc.) HT and F sold under contract, miniatures - 282,700 ('Velvet Rosaminis' most popular - 49,480). Climbers, ground cover and shrub roses - 25p each per 100, 23p per 1,000, 21p per 5,000 (prices exclude VAT, royalties and delivery)
Notcutts, SUFFOLK	HT and F (for range of colour), miniatures climbers (broad colour range, 'Mermaid' most popular), ground cover; others including China roses (3 cvs.), Rosa moyesii HT and F - 50,000, miniatures - 2,000 (most bought in from other companies), climbers - 15,000 (expect increased productivity in 1987), ground cover - 20,000, China roses - 10,000, rose 'Canary Bird' - 1,500, R. moyesii - 1,000 ~ 22p per plant + VAT, although difficult to propagate spp. and cvs. such as 'Mermaid' and R. chinensis nearer 30p
Twyford Plant Laboratories, SOMERSET	All produced under contract, therefore no data available for nos. or prices

employed. A temperature of ~ 21°C is also usually used for optimal growth.

The influence of plant growth regulators and their combination is discussed in detail by Jacobs et al (1969, 1970a, b). Cytokinin is essential for shoot multiplication, BAP being the most effective (Elliott 1970; Hasegawa, 1979, 1980). The auxin NAA is frequently incorporated into the medium in combination with BAP, although this tends to encourage the formation of callus at the base of shoot tip explants. Gibberellic acid is also often used for shoot multiplication, although the literature is again unclear on its' importance. Whilst it may increase the rate of shoot multiplication and promote shoot elongation (Bressan et al, 1981; Delbard, 1982), other reports indicate it to be inhibitory to both shoot survival and leaf expansion (Jacobs et al, 1970 a,b; Hasegawa, 1980).

The initiation of roots in vitro is stimulated by transfer to medium with a reduced concentration of MS salts (Murashige and Skoog, 1962), anything from 1/8 to 1/2 full strength. This enhancement is due to a reduction in the concentration of nitrogen salts, and a corresponding increase in the sucrose:N_2 ratio (Bressan et al, 1981; Hyndeman et al, 1982 a, b). Whilst roots may be initiated in the absence of plant growth regulators (Skirvin and Chu, 1979), an auxin or a combination of auxins is commonly added to the rooting medium. Indeed, the use of NAA is reported to give a positive correlation

between root initiation and the transplantability of rooted plantlets to soil (Hasegawa, 1980). A combination of other auxins may also be used to enhance root quality (Khosh-Khui and Sink, 1982b).

The rooting of rose shoots has been improved by increasing the irradiance level (from 1000 to 3000lux) and by the addition of low levels of activated charcoal to the medium (Bressan and Kim, 1980; Bressan et al, 1981, 1982). Although there is variation between species and cultivars, survival rates of 90-100% have been reported and there is generally little problem in the rooting and transplanting of tissue-cultured rose shoots (Hasegawa, 1980).

It is unwise therefore to define one particular regime for the general micropropagation of roses, as there may be variation in the response of different species, taxonomic sections of the genus Rosa, ploidy levels etc. (McCown, 1980; Khosh-Khui and Sink, 1982a; Sauer et al, 1985). Overall however, the rose has a relatively straightforward micropropagational pathway, making it ideal for this kind of physiological study.

CHAPTER 2.

MATERIALS AND METHODS

2.1 Initiation of explants

Two cultivars of Rosa, Iceberg and Peace were used throughout the study. Mature bushes were initially obtained from Brogdale EHS (Faversham, Kent ME13 8DX) and had been virus-tested, making them ideal as stock plant material. These were maintained in a glasshouse under natural daylight with a temperature of 17°C night/19°C day.

Nodal explants were taken and all leaves removed to allow complete sterilisation around the area of the axillary bud. Explants were surface sterilised by a 1 min. dip in 70% ethanol (+ a few drops of wetter) followed by a 5 min. dip in 6% sodium hypochlorite (+ wetter) and 3 rinses in sterile distilled water. They were then cut to ~ 2.5 cm in length to remove any tissue 'burnt' by the sodium hypochlorite, and placed into culture.

2.2 Media preparation and culture conditions

All chemicals used were obtained from either Atlas Chemical Industries Inc. USA (activated charcoal), BDH Chemicals Ltd. UK, Fisons UK, Flow Laboratories Scotland (MS), London Analytical and Bacteriological Media Ltd. UK ('lab m' agar, code MC2), or Sigma Chemical Company Ltd. USA.

Shoots were cultured on a basic salts medium, prior to experimental use, of MS (Murashige and Skoog, 1962)

with 8 μM BA (initially prepared by dissolving in a few drops of acid), 6 gl^{-1} 'lab m' agar, and 30 gl^{-1} sucrose. This cytokinin 'maintenance' medium was chosen on the recommendation of ADAS (1982). The pH was adjusted to 5.7 prior to the addition of agar and subsequent autoclave sterilisation (121°C, 1055 gcm^{-2} [≡15 psi.], 15 min.). During experiments where it was necessary to filter-sterilise sugars to prevent their caramelisation, the media without added sugars was autoclaved as before, compensating for the volume of sugar solution (20ml) to be added later. The sugar solutions were then added to the cooled medium (cool enough to be hand-held) by passing through 0.2 μM millipore filters and the whole dispensed into sterile culture jars.

Cultures were grown in 175 ml glass jars with opaque lids, containing 6-7 shoots on 30 ml of medium and were routinely subcultured every 4 weeks (=28days). These were maintained in Gallenkamp orbital incubators at 20°C ±, with an irradiance of 10 Wm^{-2} as measured at culture level inside the jar with the lid in place, and a 16h daylength. Irradiance was provided from above by warm-white fluorescent tubes. The precise media components and culture conditions for each experiment are defined within the relevant chapter.

For each experiment, new explants were multiplied up for at least 8-12 weeks to obtain sufficient shoots for experimental use. Where shoots were used which were already in culture, they were proliferated for at least 8

weeks under the new experimental conditions to enable them to acclimatise to the new environment. Any carry-over effects from a previous experiment would also thus be minimal.

2.3 Growth analyses

The rate of shoot multiplication was assessed as the number of shoots (1cm +) obtained from a single shoot explant after a 28d culture period. Similarly, vitrification (hyper-hydrated or water-soaked shoots) was assessed simply as the percentage of shoots showing this characteristic in each culture jar, each jar therefore representing one replicate.

The fresh weight increase (g) was calculated from the final fresh weight of a shoot cluster (after a 28d culture period) minus the fresh weight of the initial shoot explant. Shoots used for such growth analyses were as far as possible of a uniform initial size for different treatments within any one experiment. Any basal callus or excessively vitrified material, which often developed around the base of shoot clusters, was removed prior to weighing.

2.4 CO_2 exchange analysis and chlorophyll determination

An open system infra-red gas analyser (model 225, Analytical Development Co. Hoddeson, Herts. UK) calibrated in the differential mode, was used to measure CO_2 exhange of leaves detached from shoot clusters that

had been growing *in vitro* (Fig. 1). The plant material was maintained in a glass sample chamber at 25°C, initially in the dark to obtain a steady base line. Irradiation was provided by four 100W reflector tungsten lamps (Thorn Decorspot 95) giving 40 Wm^{-2} at the sample chamber. The rate of gas flow through the system was set at 0.8 l min^{-1}.

Leaves were removed from shoot clusters at the time of subculture, their fresh weights measured, and then placed inside the sample chamber for CO_2 exchange analysis. Each reading took ~ 5 min. per sample. Leaf areas were subsequently measured using a T&J Crump portable leaf area meter. Chlorophyll content was then determined according to Arnon (1949) by extraction in 80% acetone. Six samples were taken for each treatment, with 2 shoot clusters used for each sample. Mature leaves were taken from parent plants in the greenhouse and their CO_2 uptake and chlorophyll content measured in the same way.

The rate of carbon dioxide uptake was expressed on both a fresh weight (g) and leaf area (cm^2) basis. In order to try and eliminate the effects of growth differences between shoots of different treatments, the CO_2 uptake per unit of chlorophyll was also calculated. The units used are μmol CO_2 h^{-1} g^{-1}FW/ cm^{-2}LA/ mg^{-1} chlorophyll.

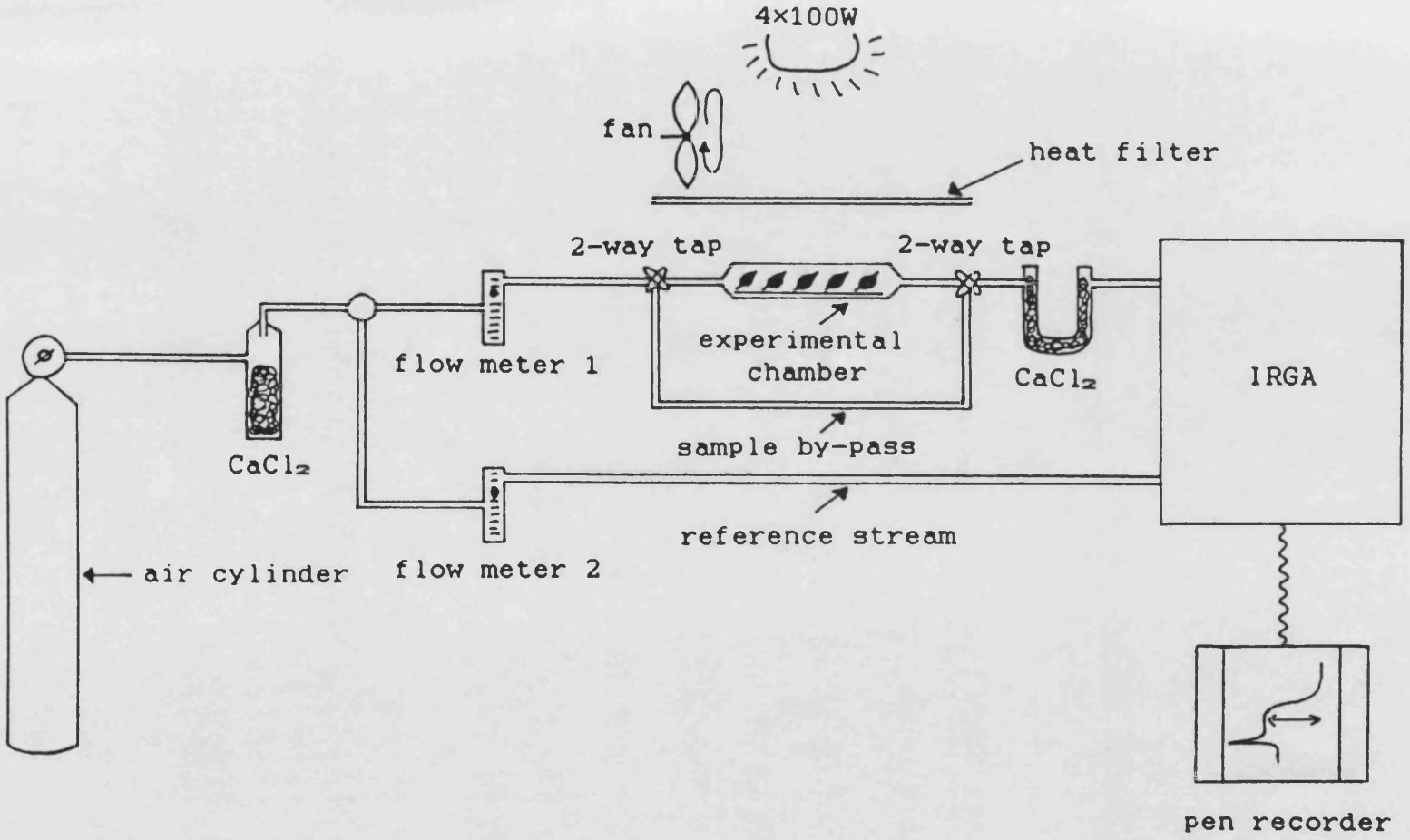


Fig. 1. A diagrammatic representation of the open system used for infra-red gas analysis (Dodge, 1986).

CHAPTER 3.

The influence of sucrose concentration on the photosynthetic ability and growth characteristics of rose shoots *in vitro*.

3.1 ABSTRACT

Reducing the concentration of sucrose included in the culture medium over successive subcultures has been tested as a method for increasing the photosynthetic (or CO_2 uptake) ability of rose shoots grown in vitro (cvs. Iceberg and Peace). Shoots maintained on 'constant' concentrations of 10, 20 and 40 gl^{-1} sucrose showed decreased rates of CO_2 uptake with increasing sucrose, although cv. Peace grew poorly at 10 gl^{-1} sucrose and showed correspondingly low rates of CO_2 uptake compared with 20 and 40 gl^{-1}. Both cultivars died when sucrose was lacking from the medium.

As sucrose was reduced in the medium from initial concentrations of 20 and 40 gl^{-1}, CO_2 uptake was found to increase, although 10 gl^{-1} sucrose seemed to be a limiting concentration, below which the growth and chlorophyll content of shoots declined. This indicates that the shoot cultures are not becoming photoautotrophic, ie. capable of maintaining growth in the absence of an added sugar when grown under standard in vitro conditions.

The development of vitrified shoots was also found to be dependent upon the concentration of sucrose, increasing significantly at 10 and 20 gl^{-1} sucrose compared with 40 and 80gl^{-1}. Rates of shoot multiplication were maximal at 40 gl^{-1}, being significantly reduced at both higher and lower sucrose.

3.2 INTRODUCTION

The effects of sugars on both photosynthesis and in particular chlorophyll levels have been well documented, although the literature generally refers to cell and callus cultures (Chaumont and Gudin, 1985). The presence of high concentrations of sucrose in the culture medium inhibits chlorophyll synthesis, so reducing the amount of chlorophyll present in plant tissues (Edelman and Hanson, 1971; Pamplin and Chapman, 1975; Dalton and Street, 1977). This effect is reversible, as transfer to lower sucrose concentrations leads to regreening of plant material. Growth is correspondingly reduced at these low concentrations of sucrose however, indicating a negative relationship between growth and chlorophyll synthesis (Barg and Umiel, 1977; Hemphill and Venketeswaren, 1978).

High sucrose concentrations also inhibit photosynthesis and photosynthetic efficiency per unit of chlorophyll (Edelman and Hanson, 1971; Herold, 1980), in vitro shoots of Douglas fir showing a potentially higher production of photosynthetic carbohydrates at lower sucrose concentrations (Evers, 1982). Similarly, photoautotrophic cells have a greater efficiency for carbon assimilation compared with photomixotrophic cells (Nishida et al, 1980; LaRosa et al, 1984).

Plant growth regulators are reported to influence the greening of plant tissue cultures (Bender et al, 1981). Cytokinins in particular, notably kinetin, have been

shown to increase chlorophyll contents and stimulate chloroplast differentiation (Stetler and Laetsch, 1965; Kaul and Sabharwal, 1971; Neumann and Raafat, 1973; Seyer et al, 1975; Yamada and Sato, 1978; Parthier, 1979; Nowak et al, 1986). Auxins too, affect chlorophyll production (Sunderland, 1966), 2,4-D causing suppression which can again be reversed by culturing onto auxin-free medium (Venketeswaren, 1965; Sunderland and Wells, 1968).

High sucrose concentrations result in a change in both the number and morphology of chloroplasts, leading to a reduced photosynthetic efficiency. It is suggested that sucrose inhibits the development of chlorophyll by acting at the stage controlling δ-aminolevulinic acid (ALA) synthesis. This is one of the early precursors in the pathway of chlorophyll synthesis, and its addition to cultures at low levels counteracts the adverse effects of sucrose to some extent (Pamplin and Chapman, 1975; Parr et al, 1976).

Other readily utilisable sugars such as glucose, fructose and maltose similarly inhibit chlorophyll synthesis in Chlorella (Shihira-Ishikawa and Hase, 1964). However it is suggested that sucrose has a direct effect on net O_2 consumption, independent of its effect on chlorophyll content. In contrast to its monosaccharide components (glucose and fructose), sucrose may directly inhibit photosynthesis or promote photorespiration (Dalton and Street, 1977).

An experiment was designed therefore to study the effect of sucrose concentration on the photosynthetic ability of rose shoots grown in vitro. An attempt to improve rates of photosynthesis was carried out by a gradual reduction in the concentration of media sucrose over successive subcultures. This is discussed with a view to making shoots independent of an exogenous sugar supply at the time of transfer to soil. The photoautotrophic culture of shoots may lead to the development of photosynthetically competent foliage which persists on transfer to soil and contributes significantly to the growth of the establishing plantlet.

3.3 MATERIALS AND METHODS

3.3.1 'Constant' and 'decreasing' sucrose

Shoot cultures of Rosa, cvs. Iceberg and Peace were cultured onto a medium of MS, 8 μM BA, 6 gl^{-1} 'lab m' agar and sucrose. The medium was prepared and sterilised by autoclaving as described previously. Cultures were maintained at 20°C with an irradiance of 10 Wm^{-2} and a 16h daylength.

Shoots initially cultured on 30 gl^{-1} sucrose were transferred to media with sucrose at concentrations of 0, 10, 20, and 40 gl^{-1}, and routinely subcultured every 4 weeks (28d). After 8 weeks (ie. for the third subculture) the concentration of sucrose supplied to 50% of the shoots was halved and in subsequent subcultures was further reduced as shown in Table 2. The remaining shoot

Table 2. Concentration of sucrose included in the medium at each subculture (1-6) to give a 'decreasing' sucrose treatment.

Initial sucrose concentration (gl^{-1})	Sucrose concentration (gl^{-1})					
	Subculture number					
	1	2	3	4	5	6
10	10	10	5	5	2.5	2.5
20	20	20	10	10	5	2.5
40	40	40	20	10	5	2.5

cultures were maintained by repeated subculturing on to 'constant' sucrose at 0, 10, 20 and 40 gl^{-1}. These concentrations of sucrose were chosen as they are within the range commonly used in <u>in vitro</u> studies.

Shoots were assessed for CO_2 uptake (IRGA) and chlorophyll content at the time of each subculture. Mature leaves from parent plants growing out in the greenhouse were also analysed for CO_2 uptake and chlorophyll content.

Six samples were taken at the end of each subculture period for both 'constant' and 'decreasing' sucrose treatments. An overall analysis of variance was made of shoots on 'constant' sucrose; the 6 samples were averaged together to give 6 replicates in total, each representing one average value for each of the 6 subculture periods.

The 'constant' and 'decreasing' sucrose treatments were compared directly at each subculture using all the data ie. the 6 sample values. They were compared using t-tests to take into account the individual variances of the two treatments.

3.3.2 Sucrose concentration and shoot growth

In a separate experiment, shoots initially cultured on 30 gl^{-1} sucrose were transferred onto media containing 10, 20, 40 or 80 gl^{-1} sucrose to observe the effect on chlorophyll content and to measure shoot growth (over 3 subcultures). The results of the two experiments are discussed together to give an overall picture of the influence of sucrose concentration on shoot growth.

3.4 RESULTS

Morphological characteristics

There were visible differences in shoot growth and development at the four sucrose concentrations. Both cultivars when cultured onto sucrose-free medium rapidly became chlorotic with little or no growth, although the larger the initial explant the better the survival. Even so, all shoots perished within 8 weeks of transfer to sucrose-free medium.

At 10 gl^{-1} sucrose, the shoots of both cultivars were small and compact, whereas at 20 and 40 gl^{-1} growth was more vigorous, with larger, more 'lush' foliage, though amounts of basal callus correspondingly increased with

increasing sucrose. At 80 gl^{-1} sucrose, shoots clusters were almost buried in callus, clearly impeding the development of healthy new shoots. Although the growth of shoots of both cultivars at 10 gl^{-1} sucrose was small and rather variable, the growth of Peace was less than Iceberg at this concentration of sucrose, in combination with a fairly high incidence of vitrification.

Both the development of vitrified shoots and the rate of shoot proliferation were significantly affected by the concentration of sucrose supplied in the medium (Fig. 2). Although levels of vitrification were generally low for all treatments, its development was found to increase with decreasing sucrose. Such shoots appeared water-soaked and translucent, with rather enlarged, brittle leaves. Similarly, the rate of shoot proliferation was decreased at 10 and 20 gl^{-1} sucrose, with maximal rates at 40 gl^{-1}. This showed a further decrease however, as sucrose was increased to 80 gl^{-1}, indicating an adverse effect of both high and low sucrose concentrations on shoot growth and proliferation.

Red anthocyanin pigment developed at 40 gl^{-1} sucrose, predominantly on leaf petioles and marginal serrations; more prominently in Iceberg than Peace.

Reducing the concentration of sucrose in the medium over successive subcultures led to a gradual reduction in the vigour and multiplication rate of shoot cultures, this again being more pronounced with shoots of Peace.

Fig. 2. The effect of sucrose concentration (gl^{-1}) on (a) vitrification and (b) the proliferation rate of rose shoot cultures, using combined data for cvs. Iceberg and Peace, based on 3 subcultures. Bar represents 5% LSD.

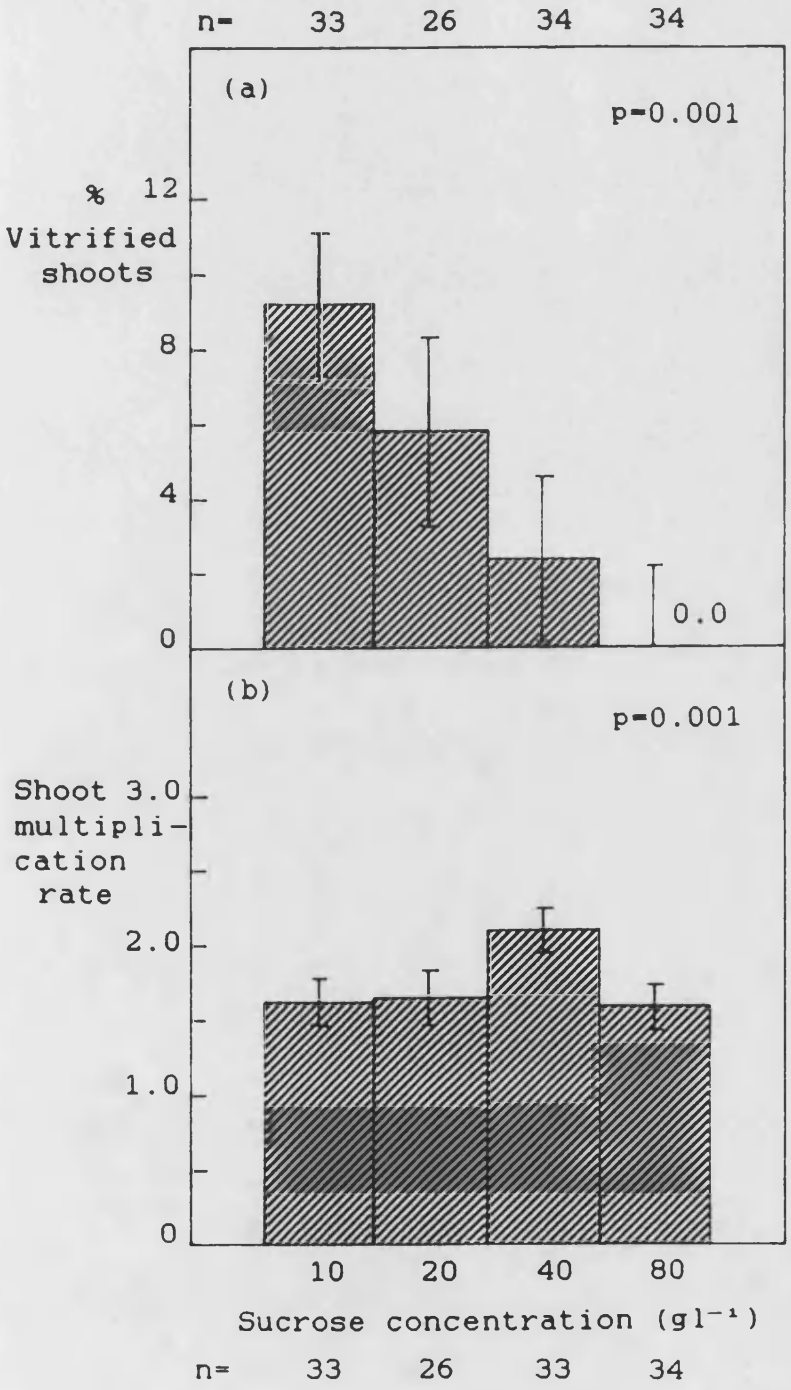
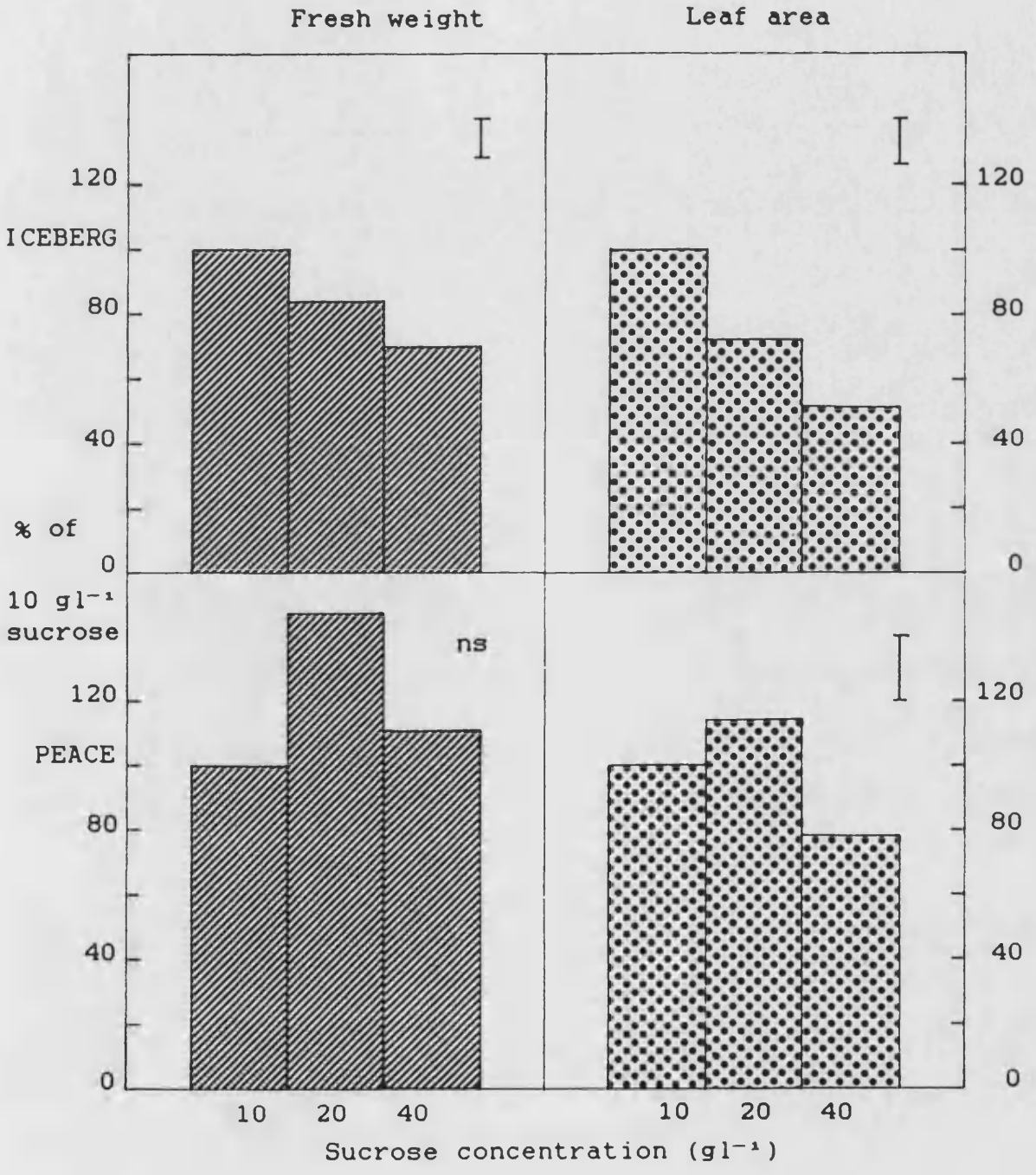


Fig. 3. CO_2 uptake by rose shoots cvs. Iceberg and Peace when cultured on media containing 10, 20 and 40 gl^{-1} sucrose. Values are expressed as a percentage of the CO_2 uptake of shoots on 10 gl^{-1} sucrose, and are based on data from 6 subcultures. Bar represents 5% LSD. (n=6)



'Constant' sucrose

The effect of culturing shoots on different, yet 'constant' concentrations of sucrose was that CO_2 uptake was reduced at 40 gl^{-1} and greatest at 10 gl^{-1} for Iceberg, for both fresh weight (FW) and leaf area (LA) data. Peace also showed a reduced rate of CO_2 uptake at 40 gl^{-1} compared with 20 gl^{-1} sucrose, but shoots grown on 20 gl^{-1} had maximal rates of CO_2 uptake not those on 10 gl^{-1} (Fig. 3). These differences were generally evident at each subculture, although the absolute values did vary over time, those for Peace at 10 gl^{-1} being particularly variable in comparison with 20 and 40 gl^{-1} sucrose (Tables 3 and 4). The CO_2 uptake of shoots on 20 and 40 gl^{-1} was therefore expressed as a percentage of that of shoots on 10 gl^{-1}, so showing the overall effect of sucrose concentration and reducing the variability of the original data (Fig. 3).

The chlorophyll content of shoots increased as sucrose was increased up to 20 gl^{-1} and although did decrease significantly for shoots of Iceberg on 40 gl^{-1} for FW data, generally remained similar at 40 gl^{-1} to the chlorophyll contents at 20 gl^{-1} sucrose (Table 5). Shoots transferred to a culture medium containing 80 gl^{-1} sucrose however, showed a visible decline in chlorophyll content, with the development of a very obvious yellow/green foliage.

Table 3. CO_2 uptake of rose shoots cv. Iceberg cultured on media containing 0, 10, 20 and 40 gl^{-1} sucrose, measured at each subculture. (n=6) (*=only 1 sample)

sucrose concentration (gl^{-1})	Subculture number					
	1	2	3	4	5	6
(a) $\mu mol\ CO_2\ g^{-1}FW\ h^{-1}$						
0	17.20	16.27	/	/	/	/
10	20.77	26.39	34.35	38.38	27.90	38.77*
20	17.46	22.39	28.26	22.17	31.98	33.62
40	11.32	16.53	26.21	19.40	23.54	37.00
5% LSD	4.12	2.75	NSD	7.08	NSD	NSD
(b) $\mu mol\ CO_2\ cm^{-2}LA\ h^{-1}$						
0	0.505	0.531	/	/	/	/
10	0.797	0.944	1.074	0.956	0.984	1.389
20	0.493	0.747	0.643	0.348	1.019	1.401
40	0.368	0.424	0.329	0.348	0.673	1.163
5% LSD	0.220	0.122	0.238	0.161	0.247	NSD

Table 4. CO_2 uptake of rose shoots cv. Peace cultured on media containing 0, 10, 20 and 40 gl^{-1} sucrose, measured at each subculture. (n=6)

sucrose concentration (gl^{-1})	Subculture number 1	2	3	4	5	6
(a) $\mu mol\ CO_2\ g^{-1}FW\ h^{-1}$						
0	22.11	5.44	/	/	/	/
10	27.75	24.85	18.79	8.32	21.10	44.00
20	29.97	18.86	28.90	19.14	42.22	49.10
40	15.58	16.77	18.58	19.41	26.90	35.30
5% LSD	5.32	7.10	7.05	5.87	11.49	NSD
(b) $\mu mol\ CO_2\ cm^{-2}LA\ h^{-1}$						
0	0.537	0.188	/	/	/	/
10	0.538	0.759	0.729	0.292	0.707	1.703
20	0.507	0.526	0.796	0.428	1.093	1.895
40	0.275	0.440	0.411	0.394	0.687	1.205
5% LSD	0.124	0.200	0.225	NSD	NSD	NSD

The chlorophyll a:b ratio of shoots on 0 and 10 gl^{-1} sucrose was reduced to ~ 2.6 and 2.8 respectively, compared with the 'normal' in vivo value of ~ 3.0 as seen at 20 and 40 gl^{-1} (Table 5).

When relating the rate of CO_2 uptake to the amount of chlorophyll present in the plant material, sucrose concentration was again found to influence the observed values (Table 5). The higher the sucrose concentration present in the medium, the less CO_2 was taken up per unit of chlorophyll, perhaps indicating a greater 'chlorophyll efficiency' at the lower concentrations of sucrose.

The CO_2 uptake of shoots growing in vitro was much less than by leaves of parent plants in vivo. The maximum values of 33.38 µmol CO_2 $g^{-1}FW$ h^{-1} for Iceberg (10 gl^{-1} sucrose at subculture 4) and 49.10 µmol CO_2 $g^{-1}FW$ h^{-1} for Peace (20 gl^{-1} sucrose at subculture 6) compared with average values of 79.82 µmol CO_2 $g^{-1}FW$ h^{-1} and 77.40 µmol CO_2 $g^{-1}FW$ h^{-1} for the two parent plants respectively (Table 6).

Mature leaves of Peace contained 2-3 times as much chlorophyll as those of Iceberg, resulting in a much reduced rate of CO_2 uptake per unit chorophyll for Peace in vivo. Cultured shoots of Iceberg contained much higher concentrations of chloropyhll compared with in vivo material, in contrast with in vitro shoots of Peace which contained less chlorophyll than in vivo material. Both cultivars however, had similar chlorophyll contents

Table 5. Chlorophyll content, a:b ratio and chlorophyll-dependent CO_2 uptake of rose shoots cvs. (a) Iceberg and (b) Peace cultured on media containing 0, 10, 20 and 40 gl^{-1} sucrose, averaged over the six 4 week subculture periods. (n=6)

	Sucrose concentration (gl^{-1})				
	0*	10	20	40	5% LSD
(a) ICEBERG					
g^{-1}FW	0.835	1.351	1.807	1.487	0.221
mg chl					
cm^{-1}LA	0.0256	0.0452	0.0508	0.0360	0.0296
a:b	2.60	2.82	3.13	3.06	0.19
µmol CO_2 mg^{-1}chl h^{-1}	21.75	23.85	15.13	14.78	5.21
(b) PEACE					
g^{-1}FW	0.474	0.951	1.786	1.702	0.277
mg chl					
cm^{-2}LA	0.0126	0.0302	0.0470	0.0431	0.012
a:b	2.58	2.78	3.24	3.11	0.20
µmol CO_2 mg^{-1}chl h^{-1}	30.91	26.02	18.05	13.95	6.75

* Figures for 0 gl^{-1} sucrose consist of the average of the first two 4 week subculture periods and are therefore excluded from the LSD calculation

Table 6. Measurements of parent plants (in June) ± standard error. (n=6)

	µmolCO_2 uptake $g^{-1}FW$	$cm^{-2}LA$	mg chlorophyll $g^{-1}FW$	$cm^{-2}LA$	a:b	µmolCO_2 mg^{-1} chl h^{-1}
ICEBERG	79.82 ±4.50	1.278 ±0.108	0.947 ±0.075	0.0147 ±0.0011	2.99 ±0.041	92.3 ±11.32
PEACE	77.40 ±4.27	1.475 ±0.088	2.237 ±0.106	0.0428 ±0.0027	3.29 ±0.071	35.3 ±2.19

in vitro when compared with each other, ~ 1.8 mg $g^{-1}FW$ and ~ 0.05 mg $cm^{-2}LA$ (at 20 gl^{-1} sucrose) (Table 5).

'Decreasing sucrose'

The cv. Iceberg was clearly better able to adapt to the reduction in media sucrose than Peace, in terms of shoot vigour, CO_2 uptake and retention of chlorophyll. Shoots of Iceberg initially growing on 40 and 20 gl^{-1} sucrose were found to significantly increase their CO_2 uptake as the concentration of sucrose was reduced, compared with shoots maintained on 'constant' sucrose (Figs. 4 and 5). Shoots of Peace initially on 40 and 20 gl^{-1} sucrose also showed an initial significant increase in CO_2 uptake as sucrose was reduced. However, when the concentration of sucrose had been dropped to less than 10 gl^{-1} (at subculture 5) the CO_2 uptake of shoots decreased to below that of shoots maintained on 'constant' sucrose (Figs. 5 and 6).

Fig. 4. Uptake of CO_2 per fresh weight by rose shoots cvs. Iceberg and Peace when grown on 'constant' (●) and 'decreasing' (o) levels of media sucrose (Table 2) assessed at each subculture. Initial sucrose concentrations were 10 gl^{-1} (a and d), 20 gl^{-1} (b and e) and 40 gl^{-1} (c and f). 'Constant' and 'decreasing' sucrose treatments compared using t-test, probability levels given. (n=6) (*= only 1 replicate)

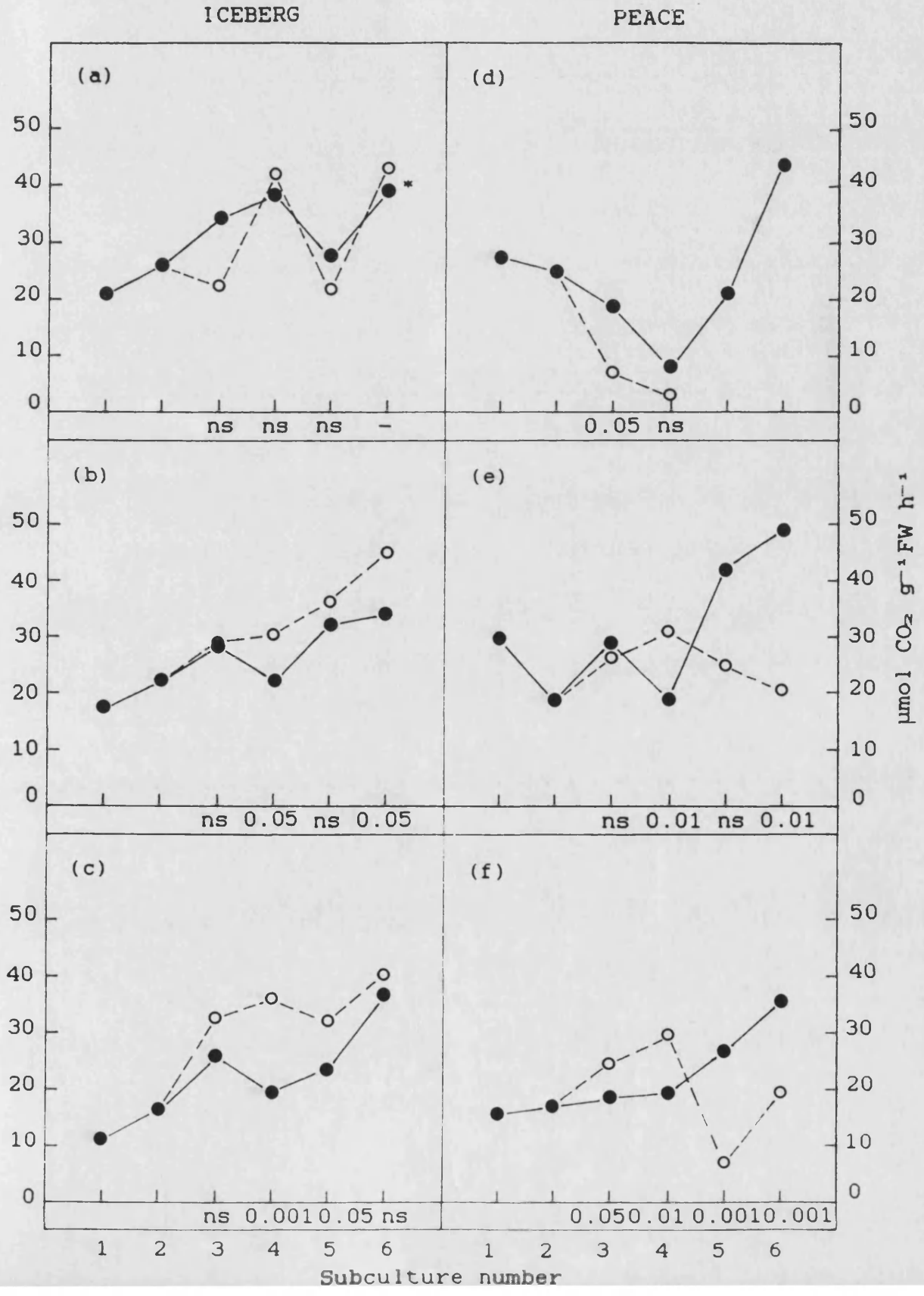
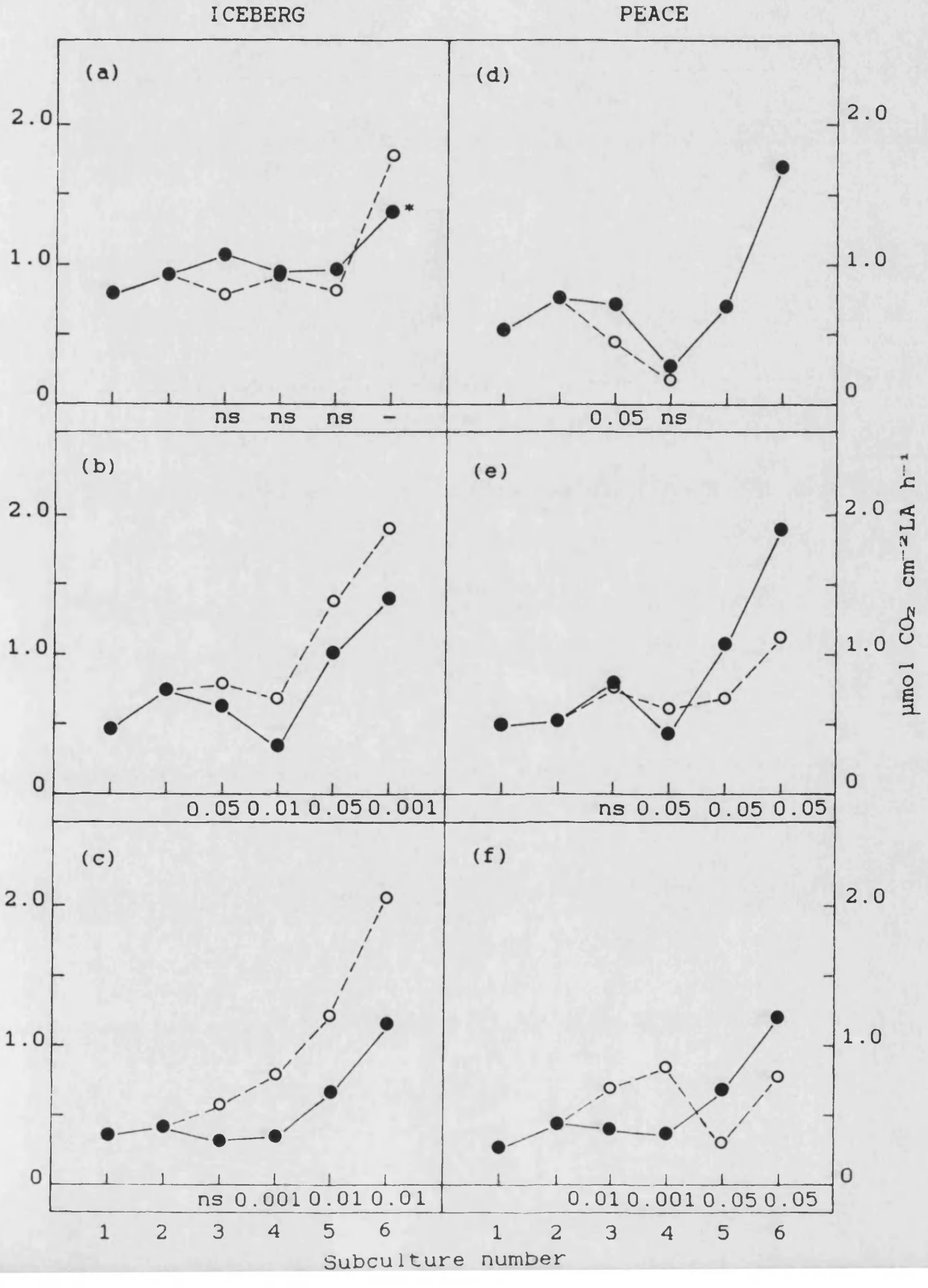


Fig. 5. Uptake of CO_2 per leaf area by rose shoots cvs. Iceberg and Peace when grown on 'constant' (●) and 'decreasing' (o) levels of media sucrose (Table 2) assessed at each subculture. Initial sucrose concentrations were 10 gl^{-1} (a and d), 20 gl^{-1} (b and e) and 40 gl^{-1} (c and f). 'Constant' and 'decreasing' sucrose treatments compared using t-test, probability levels given. (n=6) (*= only 1 replicate)



Shoots of Peace initially cultured on 10 gl^{-1} sucrose and then placed onto medium with lower concentrations of sucrose, showed reduced chlorotic growth, with a high incidence of vitrification, and died within 8 weeks. Their CO_2 uptake correspondingly declined immediately on sucrose reduction. The CO_2 uptake of Iceberg shoots treated similarly, showed no significant increase or decrease compared with shoots on 'constant' 10 gl^{-1}. The adverse effect on shoot growth was also less severe in comparison with Peace.

The effect of sucrose reduction on chlorophyll contents also differed between the two cultivars. Iceberg showed a rather variable response when initially grown on 10 gl^{-1} sucrose, but on both 20 and 40 gl^{-1} showed a slight initial increase when sucrose was reduced (Figs. 6 and 7). After the sucrose concentration had been reduced to less than 10 gl^{-1} however, chlorophyll contents declined to below those at 'constant' 20 and 40 gl^{-1} sucrose. For shoots of Peace, in contrast with Iceberg, chlorophyll concentration declined immediately on sucrose reduction, regardless of the initial concentration. This decrease became greater over time as sucrose was reduced to 10 gl^{-1} and less (Figs. 6 and 7).

The ratio of chlorophyll a:b was not greatly influenced by the reduction in the concentration of media sucrose. Shoots of Iceberg exhibited significantly decreased a:b ratios when sucrose had been dropped to 2.5 gl^{-1}, although, apart from shoots initially on 10 gl^{-1}

sucrose, the a:b ratio was never lower than ~ 2.8 (Fig. 8). Similarly, shoots of Peace initially on 20 gl^{-1} only showed significantly reduced a:b ratios when the concentration of medium sucrose had been decreased to 2.5 gl^{-1}, and the a:b ratio of shoots initially on 40 gl^{-1} sucrose showed only a slight temporary increase with respect to those of shoots maintained on 'constant' sucrose. In contrast however, the decrease in CO_2 uptake and chlorophyll content of Peace shoots on 10 gl^{-1} sucrose was also reflected by a reduction in the chlorophyll a:b ratio, down to 1.65 on a medium with 5 gl^{-1} sucrose (Fig. 8).

Carbon dioxide uptake per unit chlorophyll increased for all shoot cultures of both cultivars as the concentration of sucrose was reduced, although for neither cultivar growing on 10 gl^{-1} sucrose was the improvement a significant one (Fig. 9).

Fig. 6. Chlorophyll content per fresh weight of rose shoots cvs. Iceberg and Peace when grown on 'constant' (●) and 'decreasing' (o) levels of media sucrose (Table 2) assessed at each subculture. Initial sucrose concentrations were 10 gl^{-1} (a and d), 20 gl^{-1} (b and e) and 40 gl^{-1} (c and f). 'Constant' and 'decreasing' treatments compared using t-test, probability levels given. (n=6) (*=only 1 replicate)

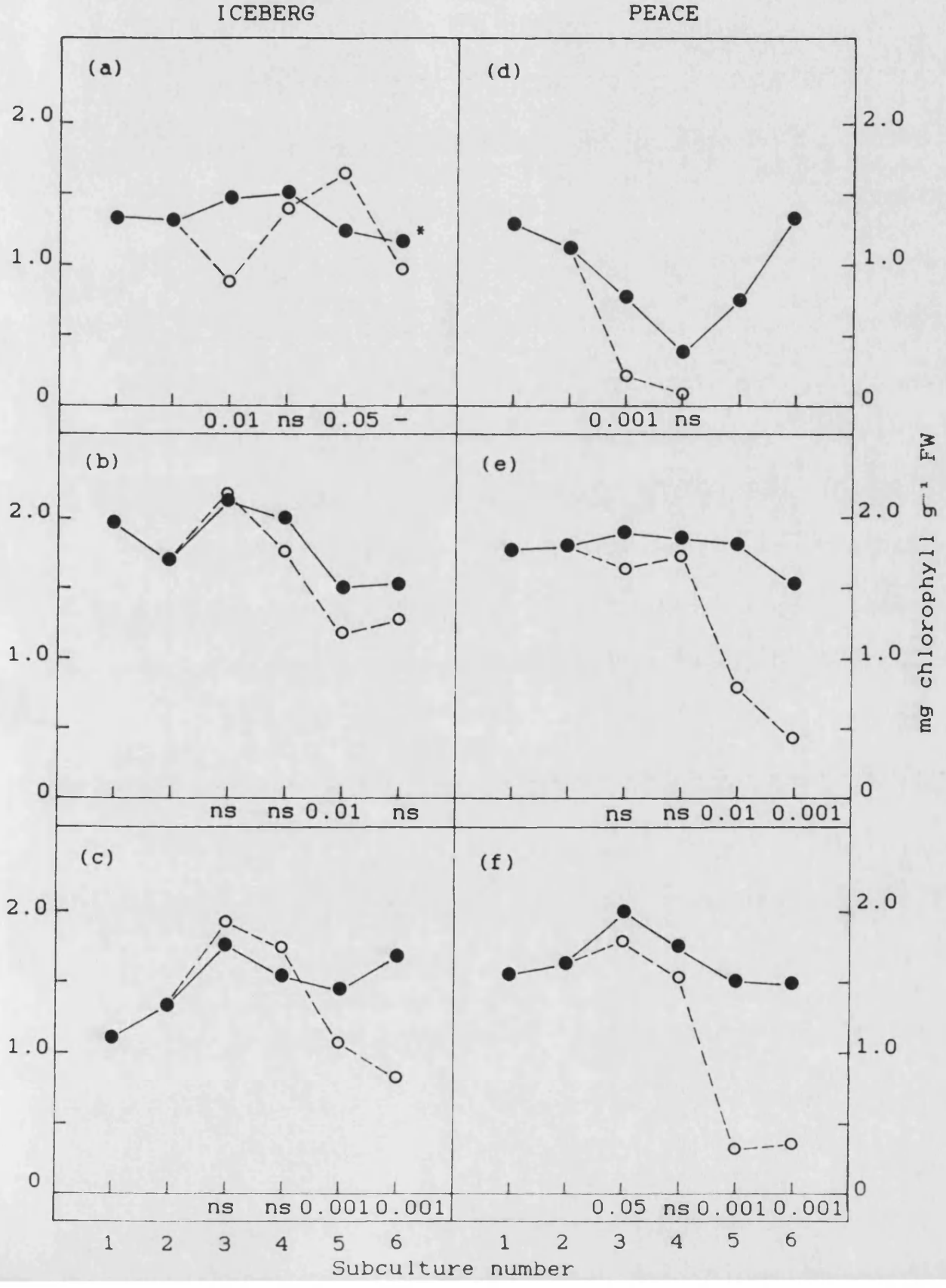


Fig. 7. Chlorophyll content per leaf area of rose shoots cvs. Iceberg and Peace when grown on 'constant' (●) and 'decreasing' (o) levels of media sucrose (Table 2) assessed at each subculture. Initial sucrose concentrations were 10 gl^{-1} (a and d), 20 gl^{-1} (b and e) and 40 gl^{-1} (c and f). 'Constant' and 'decreasing' sucrose treatments compared using t-test, probability levels given. (n=6) (*= only 1 replicate)

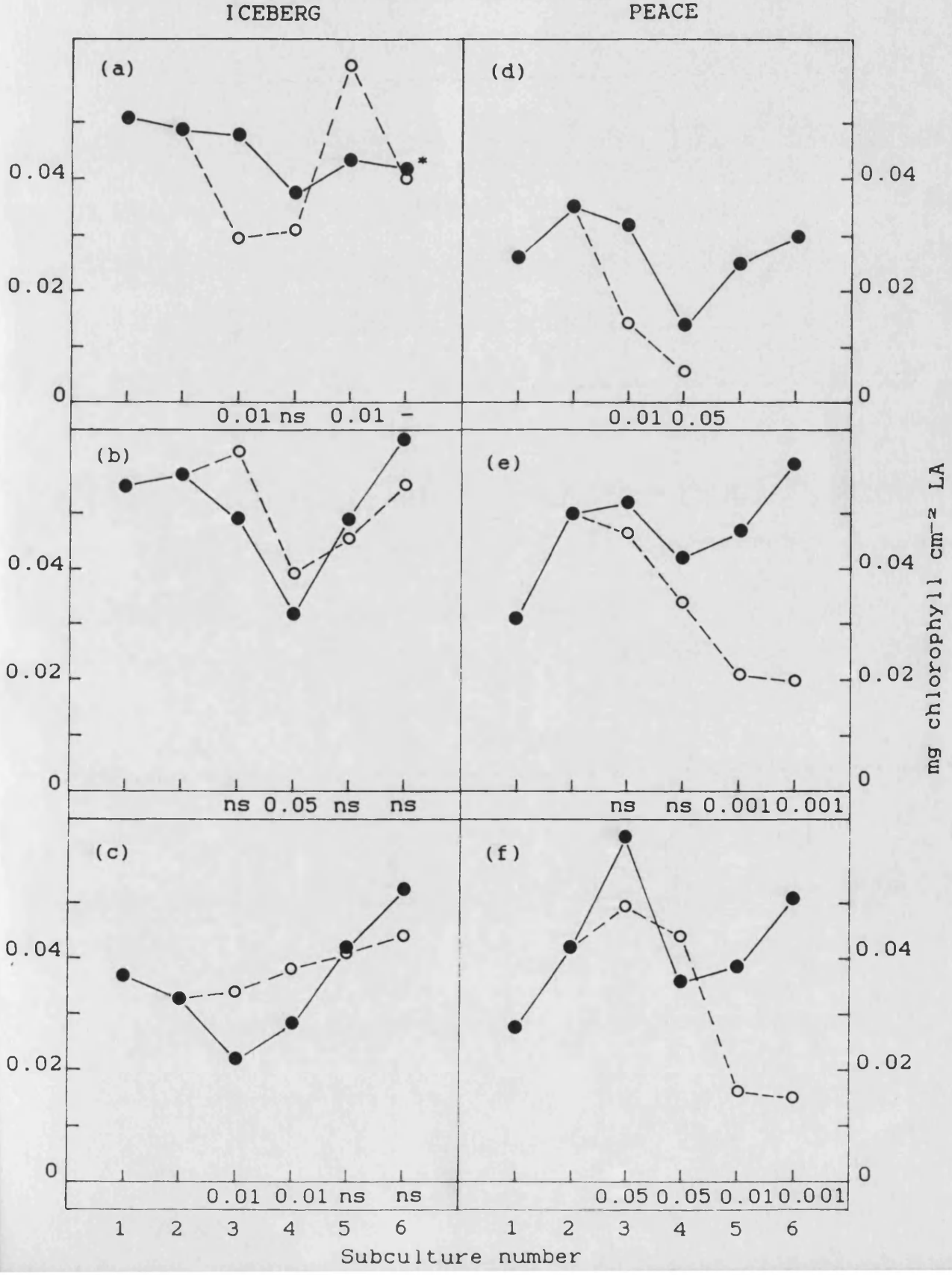


Fig. 8. Chlorophyll a:b ratio of rose shoots cvs. Iceberg and Peace when grown on 'constant' (●) and 'decreasing' (o) levels of media sucrose (Table 2) assessed at each subculture. Initial sucrose concentrations were 10 gl^{-1} (a and d), 20 gl^{-1} (b and e) and 40 gl^{-1} (c and f). 'Constant' and 'decreasing' sucrose treatments compared using t-test, probability levels given. (n=6) (*=only 1 replicate)

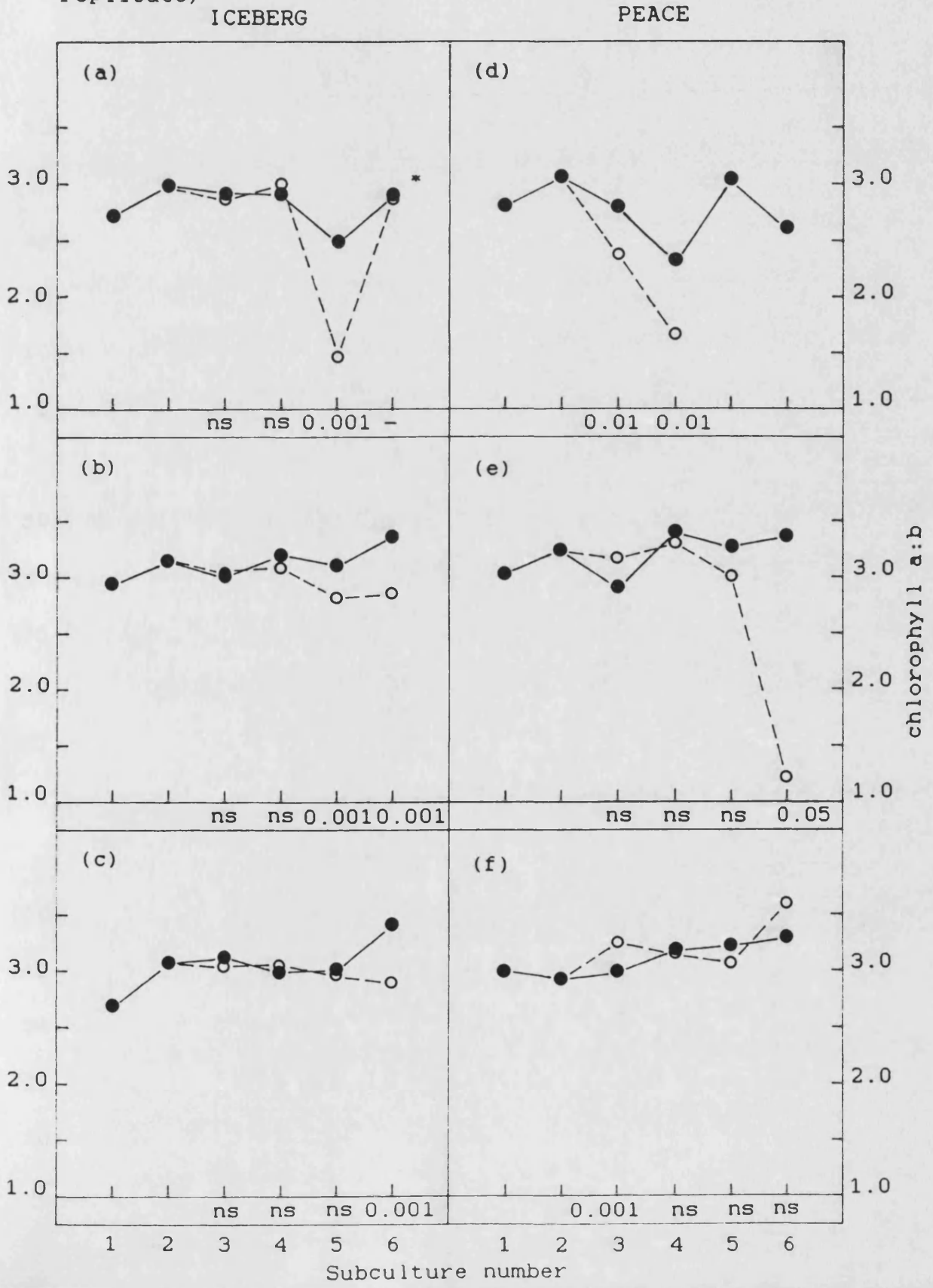
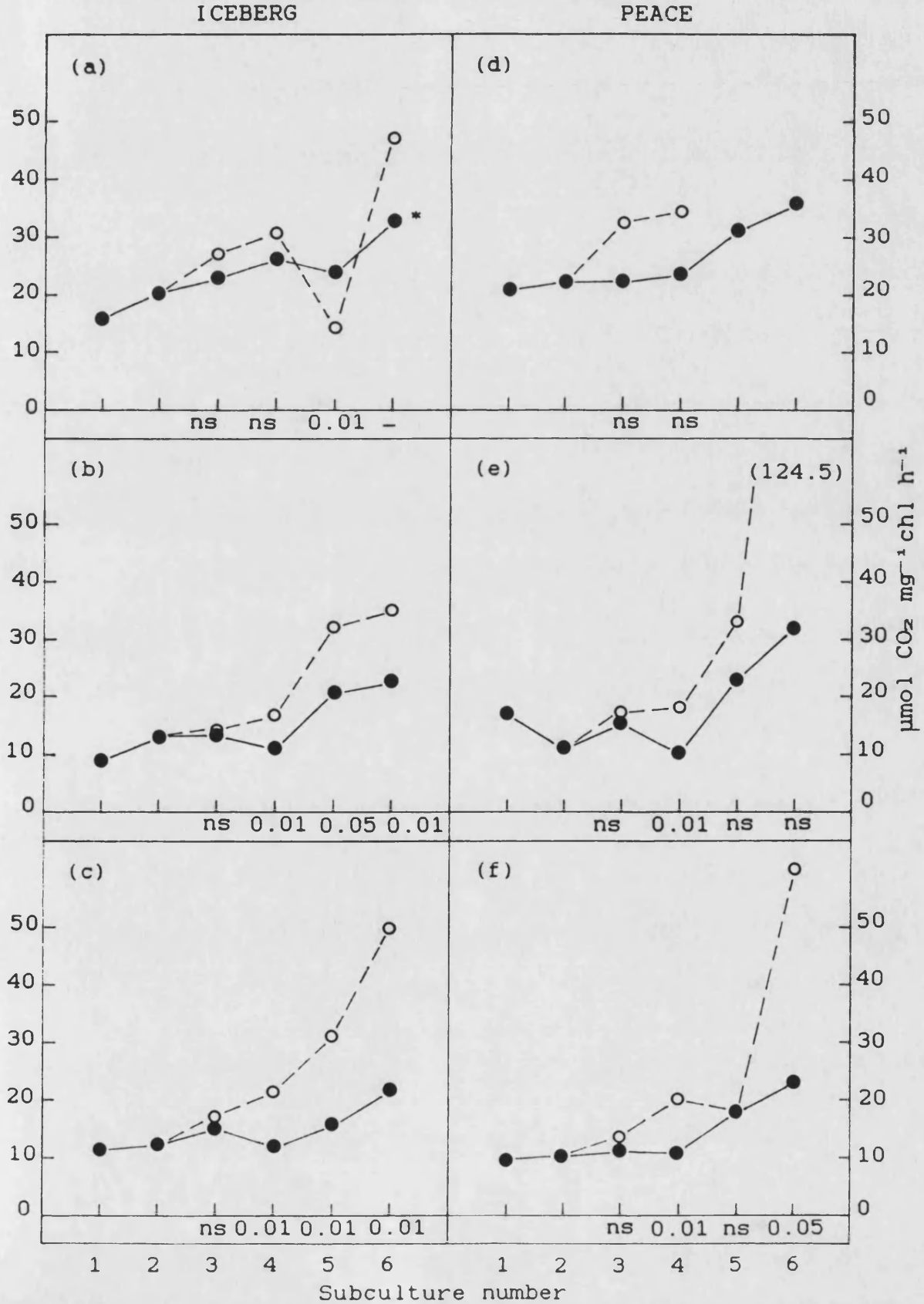


Fig. 9. Chlorophyll-dependent CO_2 uptake by rose shoots cvs. Iceberg and Peace when grown on 'constant' (●) and 'decreasing' (o) levels of media sucrose (Table 2) assessed at each subculture. Initial sucrose concentrations were 10 gl^{-1} (a and d), 20 gl^{-1} (b and e) and 40 gl^{-1} (c and f). ' Constant' and 'decreasing' sucrose treatments compared using t-test, probability levels given. (n=6) (*=only 1 replicate)



3.5 DISCUSSION

Decreasing the concentration of sucrose supplied in the culture medium appears to be a possible method for increasing the photosynthetic ability of rose shoots *in vitro*. The photosynthetic ability of leaves removed from culture, as measured by CO_2 uptake, was clearly dependent on the amount of sucrose present in the culture medium, being significantly greater at 20 gl^{-1} than 40 gl^{-1} for both cultivars. However, chlorophyll contents were not increased at the lower concentrations of sucrose, and the improvement in CO_2 uptake cannot simply be related to chlorophyll concentration alone. Indeed, Grout and Donkin (1985) indicate that carbon assimilation in shoot cultures of *Brassica* is limited by low levels of RuBP carboxylase activity ie. is due to biochemical not physical factors.

Whilst leaves from shoot cultures *in vitro* exhibited reduced rates of CO_2 activity compared with material *in vivo*, the photosynthetic rates per unit chlorophyll were also decreased *in vitro*. Similarly, microcultured shoots of *Betula platyphylla* var. Szechuanica show decreased rates of CO_2 uptake per unit chlorophyll compared with rates *in vivo*. This suggests that the 'efficiency' of chlorophyll *in vitro* is limited, possibly involving chloroplast functioning or resulting from anatomical differences evident between material *in vivo* and *in vitro* (Smith *et al*, 1986). The fact that shoots of Peace initially on 20 and 40 gl^{-1} sucrose showed

decreased rates of CO_2 uptake, even though CO_2 uptake per unit chlorophyll had increased, also suggests other, possibly biochemical factors to be limiting any further increase in the photosynthetic ability.

The differences in chlorophyll content between *in vitro* and *in vivo* material may be explained if the differences between the two parent plants are considered. Whilst leaves of Iceberg *in vitro* contained increased amounts of chlorophyll compared with *in vivo* material, those of Peace contained much less chlorophyll than *in vivo* leaves. Mature leaves of Peace however, contained nearly 3 times as much chlorophyll as those of Iceberg. This large difference between *in vivo* plants may account for the difference between *in vitro* and *in vivo* material for the two cultivars, as, when compared with each other, shoots *in vitro* contained very similar amounts of chlorophyll.

As the growing shoot is provided with sucrose in its' culture medium, it has no immediate need to photosynthesise and make its own sugars and starches. Thus it might be expected that a reduction in the concentration of sucrose included in the culture medium would result in an increased photosynthetic ability to compensate for the decrease in medium sucrose. This was found to be the case with Iceberg initially at the higher concentrations of sucrose (20 and 40 gl^{-1}), the increase in CO_2 uptake being maintained as sucrose was reduced to 5 and 2.5 gl^{-1}. Reducing the sucrose concentration from

the initial 10 gl^{-1} gave no significant increase or decrease in CO_2 uptake for either cultivar, although the CO_2 uptake of Peace shoots declined rapidly as sucrose was reduced to less than 10 gl^{-1} from the higher concentrations of 20 and 40 gl^{-1}.

A concentration of 10 gl^{-1} sucrose seems to be a limiting one, especially for Peace, below which growth and shoot development is adversely affected and so, correspondingly, is CO_2 uptake. The two cultivars respond differently at these low sucrose concentrations, this perhaps reflecting differences between the parent plants themselves. Peace is much less vigorous than Iceberg, with sturdier, stronger growth. This presumably inherent slower growth rate may be reflected in the inability of Peace shoots to thrive at low sucrose concentrations in vitro. The reduced chlorophyll contents and inferior growth of shoots at 10 gl^{-1} may prevent any increase in CO_2 uptake as sucrose concentration is reduced in the medium. In contrast, the healthy chlorophyllous growth at 20 and 40 gl^{-1} sucrose may enable shoots, in particular those of Iceberg, to increase both CO_2 uptake and 'chlorophyll efficiency' on sucrose reduction.

There does not seem to be a clear relationship between CO_2 uptake and the amount of chlorophyll present in the tissues. With Iceberg initially on media containing 20 and 40 gl^{-1} sucrose, chlorophyll contents rapidly declined as sucrose was dropped to below 10 gl^{-1}. Carbon dioxide uptake however, continued to show a

significant increase, even when chlorophyll had decreased significantly. The reduction in chlorophyll content may be compensated for by the increase in 'chlorophyll efficiency' as the concentration of sucrose is reduced, this allowing CO_2 uptake to increase further, even at decreased concentrations of chlorophyll. A similar response was seen for Peace, although here chlorophyll contents declined immediately on sucrose reduction as CO_2 uptake showed an initial improvement.

Not only was growth adversely affected at the lower concentrations of sucrose, the incidence of vitrification may also begin to cause problems and reduce the number of shoots able to survive. Vitrified or hyper-hydrated shoots do not survive the transfer to soil, soon perishing due to excessive water loss and infection (von Arnold and Eriksson, 1984). They rarely recover normal growth and therefore have to be discarded. In this study, the development of vitrified shoots did seem to be connected with the concentration of sucrose in the medium, as has been found previously (Wainwright and Marsh, 1986) and the osmotic differences between the shoot material and its growing medium may be a contributing factor (Debergh et al, 1981). The vitrification of rose shoots was rare at 40 gl^{-1} sucrose, but increased in frequency at the lower concentrations of sucrose. In contrast, the development of excessive amounts of basal callus often seen at 40 gl^{-1} sucrose, and always present at 80 gl^{-1}, may also impede healthy

shoot development and reduce rates of shoot multiplication. It is likely therefore, that a concentration of 20 or 30 gl^{-1} is optimal for the healthy growth of rose shoots *in vitro* .

Thus both high and low concentrations of sucrose seem to adversely affect shoot growth and development. These observations are supported by other reports in the literature (Pierik *et al*, 1975; Barg and Umiel, 1977; Brown *et al*, 1979). For instance, shoots of *Prunus domestica* cv. Victoria cultured onto media with a range of 0 to 60 gl^{-1} sucrose, showed reduced growth at 10 and 60 gl^{-1} after 8 weeks compared with the intermediates (Constantine, 1983). Shoots rapidly senesced and died within 4 weeks on sucrose-free medium. Rose shoots of both cultivars grown on 10 gl^{-1} sucrose in this study showed reduced chlorophyll a:b ratios compared with the value *in vivo* of ~ 3.0. This may also reflect the poor shoot growth seen at these low sucrose concentrations.

An alternative method to achieve sucrose reduction is to simply maintain cultures on the same medium for longer periods of time, although other medium components may then be depleted and become limiting. This approach was investigated with *Cinchona ledgeriana* L. shoots (Champion, 1982) which were found to be heterotrophic for the first 21d after transfer to fresh medium, during which time there was rapid uptake of sucrose by the growing shoots. As growth continued, cultures relied progressively on photoautotrophism as a source of

carbohydrate, reaching maximum photosynthetic rates after 4 weeks. In this study with Rosa, there must have been rapid depletion of sucrose from the medium after each subculture, and further investigation into the rate of sucrose depletion, together with an estimation of the sugar content of the plant material itself is required.

Jones et al (1973) report that with a suspension culture of Pogostemon cablin Benth., sucrose inversion to glucose and fructose proceeded to completion after 11d, when approximately one quarter of the total sugar had been utilised. The amount of sugar remaining after 28d, when the leaves of the rose shoot material were assessed for CO_2 uptake, will clearly affect the photosynthetic rate and needs to be further quantified.

Whilst decreasing the concentration of medium sucrose increases the photosynthetic rate of rose shoots in vitro, they are still incapable of photoautotrophic growth. The fact that shoots of both Iceberg and Peace rapidly died when cultured on sucrose-free medium indicates that these two cultivars at least, have a net negative carbon balance ie. are not photosynthetically competent (Short et al, 1984; Grout and Donkin, 1985). Efforts to induce photoautotrophic growth and hence obtain a positive carbon balance by reducing the concentration of sucrose supplied in the culture medium also proved unsuccessful, possibly due to the reduction in shoot vigour and chlorophyll contents.

Other factors of the *in vitro* environment need therefore to be studied, possibly in conjunction with sucrose manipulation, with a view to increasing the photosynthetic ability of shoots and producing photosynthetically competent foliage *in vitro*.

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CHAPTER 4.

The influence of alternative organic carbon sources (sugars) and their sterilisation on the photosynthetic ability and growth charateristics of rose shoots *in vitro*.

4.1 ABSTRACT

The fresh weight increase and shoot yield of rose shoots grown in vitro (cvs. Iceberg and Peace) were measured in response to culture on various organic carbon sources (sugars) after autoclaving or filter-sterilising. The percentage of vitrified shoots was increased on autoclaved sugar-media compared with filter-sterilised, as was the fresh weight increase of shoot cultures. Both shoot multiplication rate and fresh weight increase were significantly improved on glucose- and fructose-containing media as compared with sucrose. A glucose-medium was found to give maximal rates of shoot multiplication. All sugars were present at a concentration of 20 gl^{-1}.

The use of both maltose and fructose gave rise to shoots with increased photosynthetic rates, although shoots cultured on a maltose-medium showed an abnormal pattern of growth, thus limiting any potential use of this sugar. Shoot growth on a filter-sterilised sucrose-medium (containing only sucrose) was very poor in comparison with autoclaved sucrose, suggesting growth on the latter to be dependent upon the products of sucrose hydrolysis ie. glucose and fructose, resultant from the autoclaving process.

4.2 INTRODUCTION

Sucrose is the most commonly used source of organic carbon in the culture of plant tissues. Whilst many species do show optimal growth on sucrose-containing media (Mathes et al, 1973; Ramawat and Arya, 1977; Goyal and Arya, 1984), others thrive equally well on either sucrose or its constituent monosaccharides, glucose and fructose (Nickell and Burkholder, 1950; Hildebrandt and Riker, 1953; Vuke and Mott, 1987). Several species however, are reported to give better growth on sugars other that sucrose, eg. glucose for callus tissue of Helianthus tuberosus L. (Nitsch and Nitsch, 1956), raffinose for sugar cane cell suspension cultures (Nickell and Maretzki, 1970), fructose and glucose for cultured buds of Moris alba L. (Oka and Ohyama, 1982), sorbitol for shoot tips of some Malus cultivars (Pua and Chong, 1984, 1985) and lactose for Asparagus officinalis callus cultures (Chaumont and Gudin, 1985). It may therefore, be important and indeed useful to look at alternative sugar sources, instead of sucrose, for shoot culture and proliferation.

As well as carbon source, the area of media sterilisation and the resultant hydrolysis of media components upon autoclaving, is an important, yet neglected aspect of plant tissue culture. There are significant differences between initial pH levels and the pH measured after autoclaving. As pH is set prior to autoclaving, it should be noted that this will not be the

same as the pH of the final medium used for the culture of plant tissues (Skirvin et al, 1986). Again with the sugar component of the culture medium, the final sugar composition, following autoclave sterilisation, will not be the same as that initially included. Thirty to fifty per cent of sucrose is converted to glucose and fructose (Ball, 1953; Thorpe and Meier, 1973; Pilet and Golaz, 1985), and thus an autoclaved sucrose medium cannot be said to contain sucrose as the sole source of carbon. Indeed, media sterilised either by autoclaving or filtration can result in very different growth patterns, with material of several species giving better growth on autoclaved rather than filter-sterilised sucrose (Ball, 1953; Nitsch and Nitsch, 1956; Romberger and Tabor, 1971).

Heating sugars for some time around the melting point leads to sugar pyrolysis and a corresponding browning or caramelisation of the medium (Peer, 1971). This amber colouration is particularly strong in fructose-containing media. Autoclaving fructose also results in the production of compounds which are toxic to many species, reducing or even preventing growth (Stehsel and Caplin, 1969; Redei, 1974; Nash and Boll, 1975; Davis et al, 1977). The filter-sterilisation of sugars prevents both their hydrolysis and caramelisation and may be of interest in the study of plant growth in culture.

The following set of experiments report the effect of various carbon (sugar) sources and their sterilisation by

either autoclaving or sterile filtration, on the photosynthetic ability and growth characteristics of rose shoot cultures in vitro.

4.3 MATERIALS AND METHODS

4.3.1 Alternative carbon (sugar) sources

Nodal explants were initiated into culture onto a medium of MS, 8 μM BA, 6 gl^{-1} 'lab m' agar and one of 5 sugars at concentrations of 10 and 20 gl^{-1}. These 5 sugars were sucrose, maltose, D-fructose - low in glucose, D-glucose anhydrous (dextrose) and an equal combination of glucose and fructose. All fructose-containing media were sterilised by filtration (ie. the sugar solutions were filtered and added to pre-autoclaved medium), as fructose was found to caramelise rapidly on autoclaving and the resulting media failed to set. All other media were autoclaved.

Shoots were maintained on these media for three 4 week (28d) subculture periods, and were assessed for CO_2 uptake (IRGA) and chlorophyll content at the end of each culture period. The data obtained at each subculture (6 samples) was combined to give an overall analysis of variance with 18 replicates for each treatment (6 samples, 3 culture periods). Measurements of vitrification and shoot multiplication rate were also made.

4.3.2 Alternative sugars and their sterilisation

Explants were initiated into culture onto a medium of MS, 8 μM BA, 6 gl^{-1} 'lab m' agar and one of 4 sugars at 20 gl^{-1}; sucrose, glucose, fructose and an equal combination of glucose and fructose. Media was sterilised in either of two ways, by autoclaving the complete medium including sugars, or by filter-sterilising the sugars separately and adding them to the rest of the pre-autoclaved medium.

Shoots were cultured on these 8 different media for three 4 week (28d) subculture periods, and were assessed for CO_2 uptake and chlorophyll content. The data was combined for an overall 2-way analysis of variance with 18 replicates for each treatment. Measurements of vitrification, shoot multiplication rate and fresh weight increase were also made.

4.4 RESULTS

4.4.1 Alternative carbon (sugar) sources

Growth characteristics

The overall growth patterns are described in Table 7. Explants of both cultivars showed poor growth and low rates of proliferation when cultured on media with 10 gl^{-1} of sugar, and were therefore discarded after 8 weeks. Optimal growth was shown by shoots cultured on a glucose+fructose-containing medium, in terms of leaf size and lushness of growth etc. Maltose gave rise to a highly

Table 7. Growth characteristics of rose shoots cvs. Iceberg and Peace cultured on media containing various sugars (at 20 gl^{-1}).

Sugar type	ICEBERG	PEACE
SUCROSE	Generally small. Foliage green and healthy, although some yellowing of older leaves. Anthocyanins present. Not as lush as glucose + fructose	Average growth, not particularly lush
MALTOSE	Generally small, bases of shoot clusters often black and leaves curled under. Chlorotic patches at base of leaflets, rest of leaf green. Basal callus	Compact growth, some shoot clusters green, others similar to cv. Iceberg ie. black bases, curled leaves etc. No proliferation of such shoots
GLUC + FRUC	Good healthy growth with lush green foliage. Anthocyanins present	Slow growth, but overall green and healthy
GLUCOSE	Generally good growth with large leaves. Green and healthy foliage, although some slight yellowing. Basal callus and anthocyanins present	Slow growth, leaves often curled under and slightly 'crisp'. Overall, green foliage with some slight yellowing
FRUCTOSE	Small shoots with healthy large green leaves. Not as lush as glucose or glucose + fructose	Small growth with some chlorosis on older leaves. Some improvement over time, with the development of large healthy leaves. Smaller shoots appear 'crisp'

Fig. 10. Percentage of vitrified rose shoots developing on sugars at 10 and 20 gl^{-1}, combining the data for cvs. Iceberg and Peace. Bar represents 5% LSD. (n=10)

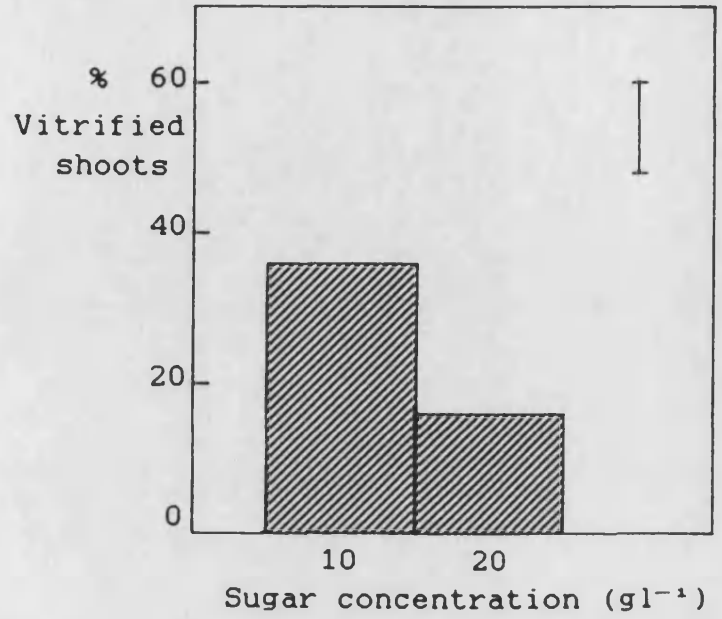
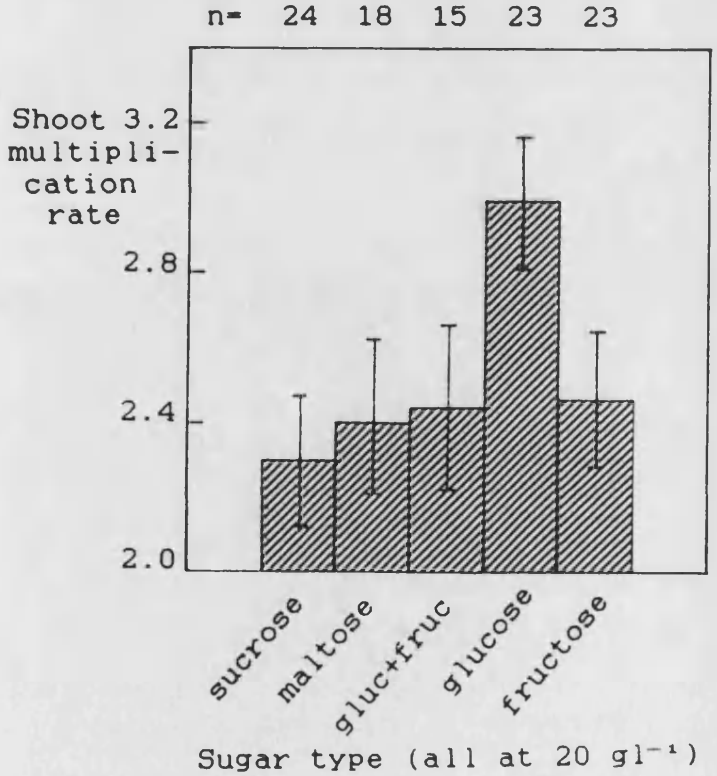


Fig. 11. Rates of shoot multiplication for rose shoots cultured on media containing various sugars (at 20 gl^{-1}), combining the data for cvs. Iceberg and Peace. Bar represents 5% LSD



abnormal pattern of growth, resulting in a low multiplication rate for such shoot cultures.

Measurements of vitrification were taken for shoots grown on all sugars at both 10 and 20 gl^{-1}. A 2-way anovar on the combined Iceberg and Peace data indicated no significant difference between the 5 sugars and no significant interaction between sugar type and its concentration. A concentration of 10 gl^{-1} however, gave rise to twice as many vitrified shoots as developed at 20 gl^{-1} ($p=0.001$) (Fig. 10).

The multiplication rate of shoots cultured on a glucose-containing medium was significantly greater than those of shoots on any of the other 4 sugars (Fig. 11), a sucrose-containing medium giving the lowest rate of multiplication, ~ 2.30 compared with 2.98 for glucose.

Physiological characteristics

The type of sugar used for shoot culture had a significant effect on the photosynthetic ability of rose shoots. For Iceberg, shoots grown on maltose- and fructose-containing media showed significantly improved rates of CO_2 uptake, those on a maltose-medium having maximal rates of 32.70 μmol CO_2 $g^{-1}FW$ h^{-1}. Shoots of Peace also showed increased photosynthetic rates on maltose- and fructose-media, although for only maltose was the increase a significant one (Table 8).

Table 8. CO_2 uptake by rose shoots cvs.(a) Iceberg and(b) Peace cultured on media containing various sugars (at 20 gl^{-1}). (n=18 unless otherwise stated)

µmol CO_2 h^{-1}	sucr	malt	gluc + fruc	gluc	fruc	5% LSD	
(a) ICEBERG							
g^{-1}FW	22.27	32.68	25.51	21.38	30.83 (n=14)	6.52 (n=18)	7.39 (n=14)
cm^{-2}LA	0.776	1.591	0.973	0.740	1.113	0.242	0.275
(b) PEACE							
g^{-1}FW	20.99 (n=12)	33.38	21.65	20.50	24.53	6.08 (n=18)	7.44 (n=12)
cm^{-2}LA	0.694	1.309	0.847	0.803	0.927	0.208	0.255

Chlorophyll contents were not significantly affected by the type of sugar, except for Iceberg FW data, which indicated a significant decrease in chlorophyll for shoots on a maltose-containing medium. This was not confirmed by the LA data however. Maltose- and fructose-media gave increased chlorophyll contents for shoots of Peace, although this increase was not found to be significant (Table 9).

The chlorophyll a:b ratio and CO_2 uptake per unit chlorophyll were both influenced by the type of sugar (Table 9). Tha a:b ratio of shoots of Iceberg grown on a maltose-medium was significantly increased compared with the 4 other sugars, although shoots on all sugars possessed a:b ratios which were higher than the 'normal'

Table 9. Chlorophyll content, a:b ratio and chlorophyll-dependent CO_2 uptake of rose shoots cvs.(a) Iceberg and (b) Peace cultured on media containing various sugars (at 20 gl^{-1}). (n=18 unless otherwise stated)

	sucr	malt	gluc + fruc	gluc	fruc	5% LSD	
(a) ICEBERG							
mg chl g^{-1}FW	1.566	1.172	1.409	1.405	1.584 (n=14)	0.157 (n=18)	0.178 (n=14)
mg chl cm^{-2}LA	0.0558	0.0579	0.0559	0.0503	0.0585	NSD	
a:b	3.33	3.77	3.10	3.36	3.44	0.34	0.38
µmolCO_2 mg^{-1}chl h^{-1}	13.98	29.62	18.51	14.77	19.56	4.73	5.37
(b) PEACE							
mg chl g^{-1}FW	1.188 (n=12)	1.485	1.275	1.239	1.460	NSD	
mg chl cm^{-2}LA	0.0440	0.0607	0.0496	0.0475	0.0600	NSD	
a:b	3.30	2.46	2.77	2.70	2.96	0.36 (n=18)	0.44 (n=12)
µmolCO_2 mg^{-1}chl h^{-1}	16.53	27.55	17.66	16.97	18.91	6.73	8.24

value of ~ 3.0. For shoots of Peace, a sucrose-medium gave rise to shoots with maximal a:b ratios (3.30), shoots on all other sugars except fructose (2.96) having a:b ratios decreased in comparison with the 'normal' 3.0;

shoots on a maltose-medium showing a significantly decreased a:b ratio of 2.46. For both cultivars, the CO_2 uptake per unit chloropyhll was maximal on maltose-containing medium, being 29.62 µmol CO_2 mg^{-1} chlorophyll h^{-1} and 27.50 µmol CO_2 mg^{-1} chlorophyll h^{-1} for Iceberg and Peace respectively. This compared with 13.98 µmol CO_2 mg^{-1} chlorophyll h^{-1} and 16.50 µmol CO_2 mg^{-1} chlorophyll h^{-1} for shoots on sucrose-medium.

4.4.2 Alternative sugars and their sterilisation

Growth characteristics

Both the type of sugar and the method of sterilisation were seen to affect the growth patterns of rose shoot cultures (Table 10). Shoot cultures of Peace were generally more compact than Iceberg, the latter cultivar frequently developing red anthocyanin pigment on leaves and petioles. Shoots of both cultivars showed superior growth on glucose- and/or fructose-containing media, in comparison with a sucrose-medium. The development of basal callus was evident with all 4 sugars used in this study, although was present in particularly large amounts on a filter-sterilised sucrose-medium. The shoots on filter-sterilised sucrose were also rather poor and chlorotic in their growth, particularly for Peace.

A 2-way anovar for % vitrification, shoot multiplication rate and fresh weight increase indicated differences between sugar type and the method of

Table 10. Growth characteristics of rose shoots cvs. Iceberg and Peace cultured on media with various sugars (at 20 gl^{-1}) sterilised either by autoclaving (A) or by sterile-filtration (F).

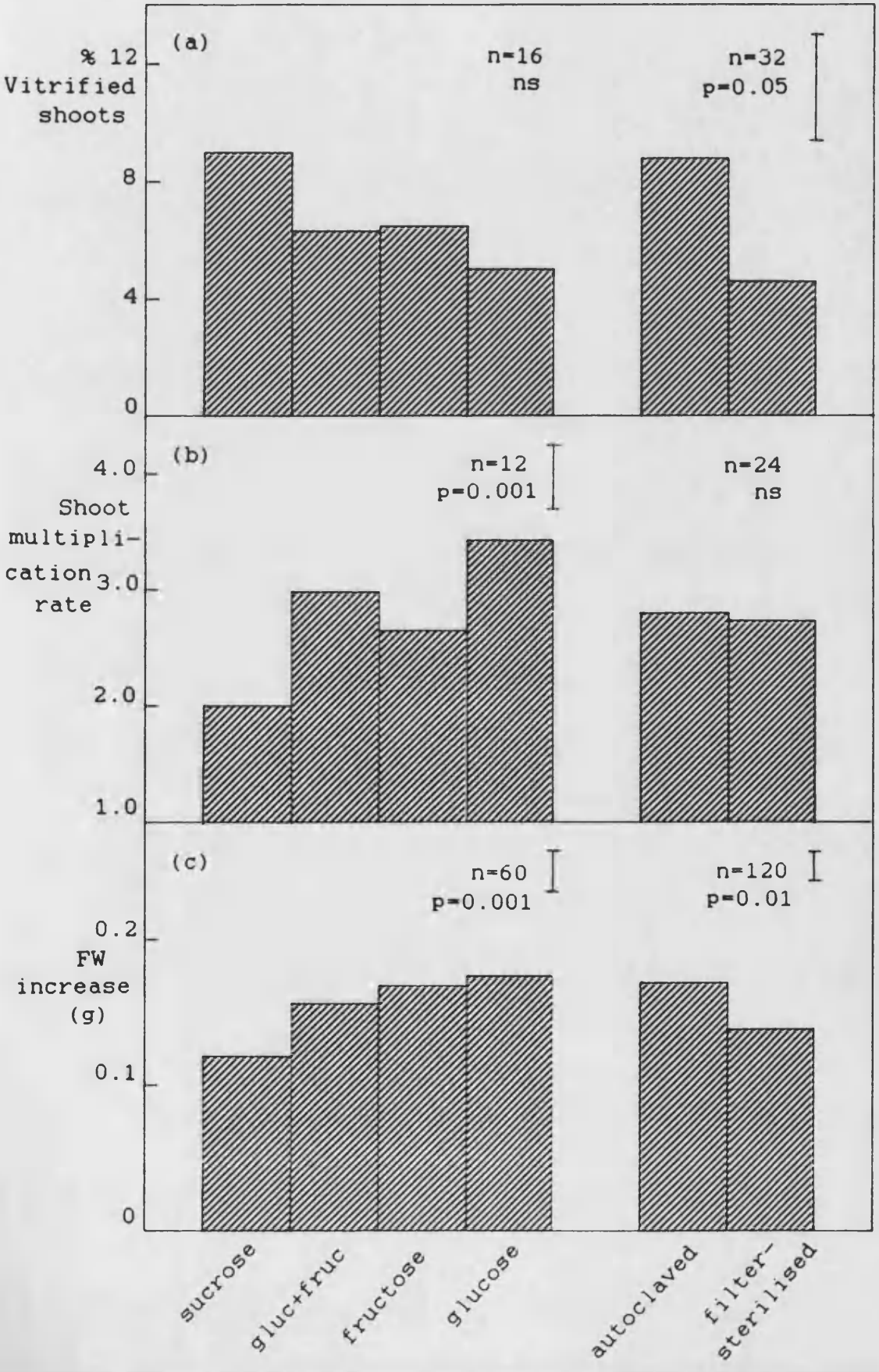
Sugar		ICEBERG	PEACE
SUCROSE	A	Compact growth, slight chlorosis. Fair amount of basal callus	Small, compact growth, overall very poor. All rather chlorotic, older leaves yellow
	F	Small growth, rather chlorotic and variable. Large amounts of basal callus	Small growth, chlorotic and very poor. Very prominent basal callus. Growth improved over time, although remaining small.
GLUC+FRUC	A	Good green growth. Often vitrified material at base- due to sloppy texture of medium? Anthocyanins present and basal callus	Average growth. Green foliage, but with some chlorosis. Growth improved over time, becoming more lush and green. Basal callus
	F	Good lush green growth, better than g+f A. Some anthocyanin and basal callus present	Good lush green compact growth, with some slight yellowing. Some leaves rather 'crisp'. Basal callus
FRUCTOSE	A	Small, rather varied growth. Generally green foliage, but with darker central leaves	Compact growth. Green foliage, with some chlorosis at leaf bases. Leaves often 'crisp' and curled. Very little basal callus
	F	Good lush green growth with large leaves. Some slight chlorosis. Anthocyanin pigments present	Overall good growth with lush foliage. Generally green, although some yellowing. Basal callus
GLUCOSE	A	Good lush growth, with some slight yellowing. Bluish 'bloom' on leaves. Anthocyanins present	Compact green growth, although some slight yellowing. Some 'crisp' leaves. Basal callus
	F	Overall good lush green growth, although some slight yellowing. Anthocyanins present	Compact growth. Overall good green foliage, with some slight yellowing. Basal callus

sterilisation, but no significant interaction between the two. The incidence of vitrification was generally low for both cultivars, with a maximum of 9% of shoots on sucrose-media being vitrified. The type of sugar had no significant effect on vitrification, although the method of sterilisation clearly did (Fig. 12). The percentage of vitrified shoots was nearly doubled on media sterilised by autoclaving, being 8.8% as compared with 4.6% on filter-sterilised sugar-media ($p=0.05$).

The rate of shoot multiplication was significantly influenced by sugar type, but not by the method of sterilisation (Fig. 12). Shoots grown on glucose-containing media had maximal multiplication rates, ~ 3.4 compared with 2.0 for sucrose-media. Indeed, shoots grown with glucose and/or fructose as carbon source all showed multiplication rates significantly improved with respect to rates on sucrose-media ($p=0.001$).

The fresh weight increase of shoot cultures showed a similar pattern to shoot multiplication rate in response to sugar type. Glucose- and fructose-containing media again gave improved shoot growth compared with sucrose-media (Fig. 12). The method of sterilisation also had a significant effect on the fresh weight increase, shoots cultured on autoclaved media showing a greater fresh weight increase than on filter-sterilised media ($p=0.01$).

Fig. 12. The effect of sugar type and sterilisation on (a) vitrification, (b) shoot multiplication rate and (c) the fresh weight increase of rose shoot cultures, combining the data for cvs. Iceberg and Peace. Bar represents 5% LSD, probability levels given.



Physiological characteristics

Neither sugar type nor the method of sterilisation had a significant effect on the CO_2 uptake ability of shoots of Iceberg, although shoots of Peace grown on glucose- and/or fructose-containing media all showed significantly increased rates of CO_2 uptake compared with those on sucrose (Tables 11 and 12). Chlorophyll contents however, were altered in response to sugar type. Shoots of Iceberg cultured on fructose-media contained significantly increased amounts of chlorophyll, 1.753 mg g^{-1}FW compared with an average of 1.560 mg g^{-1}FW for the other 3 sugars. Peace showed increased chlorophyll contents on all glucose- and/or fructose-containing media compared with sucrose. Shoots of Iceberg on autoclaved sucrose-medium also contained increased amounts of chlorophyll compared with filter-sterilised sucrose, thus verifying the differences that were clearly visible.

The chlorophyll a:b ratio of shoots of Iceberg was ~ 3.0 for all sugars, although the a:b ratio of shoots on glucose+fructose media was significantly greater (3.15) than those on sucrose (2.88). Similarly, the chlorophyll a:b ratio of Peace shoots on sucrose-media was significantly less than those of shoots on the other sugars, being 2.61 compared with ~ 3.0 for the glucose- and/or fructose-media (Tables 11 and 12).

Table 11. CO_2 uptake, chlorophyll content, a:b ratio and chlorophyll-dependent CO_2 uptake by rose shoots cv. Iceberg cultured on media containing various sugars (at 20 gl^{-1}) sterilised either by autoclaving (A) or by sterile-filtration (F).

	µmol CO_2 g^{-1}FW h^{-1}			µmol CO_2 g^{-1}LA h^{-1}		
	A	F	Mean	A	F	Mean
sucr	24.11	25.36	24.73	0.837	0.885	0.861
g+f	28.43	24.49	26.46	0.993	0.833	0.913
fruc	28.56	23.02	25.79	0.941	0.492	0.866
gluc	25.55	24.70	25.12 (n=36) NSD	0.789	0.880	0.834 (n=36) NSD
Mean	26.66	24.39	(n=72) NSD	0.890	0.847	(n=72) NSD
	$c_2 * c_3$ (n=18)		NSD	$c_2 * c_3$ (n=18)		NSD

	mg chlorophyll g^{-1}FW			mg chlorophyll cm^{-2}LA		
	A	F	Mean	A	F	Mean
sucr	1.675	1.408	1.541	0.0573	0.0470	0.0522
g+f	1.497	1.670	1.584	0.0518	0.0568	0.0543
fruc	1.706	1.800	1.753	0.0574	0.0620	0.0597
gluc	1.603	1.499	1.551 5% LSD =0.151	0.0491	0.0540	0.0515 5% LSD =0.0050
Mean	1.620	1.594	NSD	0.0539	0.0549	NSD
	$c_2 * c_3$	5% LSD=0.214		$c_2 * c_3$	5% LSD=0.0071	

	chlorophyll a:b			µmol CO_2 mg^{-1}chl h^{-1}		
	A	F	Mean	A	F	Mean
sucr	3.01	2.75	2.88	14.86	18.75	16.80
g+f	3.21	3.09	3.15	19.06	15.05	17.06
fruc	2.94	3.00	2.97	16.98	12.85	14.92
gluc	2.89	2.98	2.94 5% LSD =0.11	15.96	16.26	16.11 NSD
Mean	3.01	2.95	NSD	16.72	15.73	NSD
	$c_2 * c_3$	5% LSD=0.16		$c_2 * c_3$	5% LSD=2.90	

Table 12. CO_2 uptake, chlorophyll content, a:b ratio and chlorophyll-dependent CO_2 uptake by rose shoots cv. Peace grown on media containing various sugars (at 20 gl^{-1}) sterilised either by autoclaving (A) or by sterile-filtration (F).

	µmol CO_2 g^{-1}FW h^{-1}			µmol CO_2 cm^{-2}LA h^{-1}		
	A	F	Mean	A	F	Mean
sucr	18.62	20.02	19.33	0.683	0.692	0.688
g+f	27.03	26.81	26.92	1.052	0.924	0.988
fruc	26.94	27.88	27.41	1.114	1.021	1.067
gluc	25.80	27.97	26.89	0.941	0.949	0.948
			(n=36)			(n=36)
		5% LSD=2.87			5% LSD=0.096	
Mean	24.60	25.68	(n=72)	0.948	0.896	(n=72)
			NSD			NSD
	$c_2 * c_3$ (n=18)		NSD	$c_2 * c_3$ (n=18)		NSD

	mg chlorophyll g^{-1}FW			mg chlorophyll cm^{-2}LA		
	A	F	Mean	A	F	Mean
sucr	1.278	1.208	1.243	0.0479	0.0419	0.0449
g+f	1.590	1.603	1.596	0.0623	0.0576	0.0599
fruc	1.737	1.676	1.706	0.0740	0.0619	0.0679
gluc	1.452	1.462	1.457	0.0530	0.0508	0.0519
			5% LSD			5% LSD
			=0.126			=0.0049
Mean	1.514	1.487	NSD	0.0593	0.0530	5% LSD
						=0.0069
	$c_2 * c_3$		NSD	$c_2 * c_3$		NSD

	chlorophyll a:b			µmol CO_2 mg^{-1}chl h^{-1}		
	A	F	Mean	A	F	Mean
sucr	2.65	2.58	2.61	14.77	17.34	16.06
g+f	3.07	3.13	3.10	16.98	16.93	16.96
fruc	3.15	3.08	3.12	15.66	17.00	16.33
gluc	2.93	2.88	2.90	17.90	19.34	18.62
			5% LSD			5% LSD
			=0.07			=1.88
Mean	2.95	2.92	NSD	16.33	17.65	NSD
	$c_2 * c_3$		NSD	$c_2 * c_3$		NSD

The method of sugar sterilisation had no significant effect on the chlorophyll a:b ratio or the CO_2 uptake per unit chlorophyll of shoots. There were differences however, in the CO_2 uptake per unit chlorophyll between shoots on the 4 different sugar sources. For Iceberg, the 'chlorophyll efficiency' of shoots on autoclaved sucrose was significantly lower than for filter-sterilised sucrose, whereas for both glucose+fructose- and fructose-media, 'chlorophyll efficiency' was greater for shoots on the autoclaved media. For shoots of Peace, a glucose-medium gave maximal rates of CO_2 uptake per unit chlorophyll, 18.62 μmol CO_2 mg^{-1} chlorophyll h^{-1} compared with an average of 16.45 μmol CO_2 mg^{-1} chlorophyll h^{-1} for the other 3 sugars.

4.5 DISCUSSION

Both the type of sugar and the method by which it is sterilised have significant effects on the growth and development of rose shoots *in vitro*. Although the experimental data gives a large array of results, it may be possible to draw some conclusions and suggest further ideas for improved culture. The only chemical difference between autoclaved and filter-sterilised sugar-media is in the sterilisation of the sugars themselves. All other medium components have undergone autoclave sterilisation and thus any differences in shoot growth may be assumed to be due to differences in sugar content resulting from the sterilisation technique. Autoclaving sugars will lead to the formation of hydrolysis products eg. glucose and fructose from sucrose, whereas the sugars will remain 'intact' ie. unaltered when sterilised by filtration.

Shoots of both Iceberg and Peace showed superior growth on glucose- and/or fructose-containing media, in particular when cultured on a medium containing an equal combination of glucose and fructose. The culture of shoots on a maltose-medium gave rise to a highly abnormal pattern of growth, with blackening of the basal portion of the explant in contact with the medium, and curled leaves with chlorotic patches. Smith (1932) reports that autoclaving maltose at 15 lbs. pressure results in the hydrolysis of maltose to give glucose, and slow destruction of the glucose with the production of acid. This acid may be responsible for the abnormal growth and

blackening of explants observed when shoots were cultured on an autoclaved maltose-medium.

There was no obvious difference between shoot growth on autoclaved as opposed to filter-sterilised sugar media, although the latter often gave rise to more lush vigorous growth. This was with the notable exception of sucrose-media however, shoot growth of both cultivars being superior on autoclaved rather than filter-sterilised sucrose, with the large amounts of basal callus which developed on filter-sterilised sucrose impeding healthy shoot growth. Shoots cultured on autoclaved fructose-media showed no apparent reduction in growth contrary to other reports in the literature indicating some species to be severely inhibited by the products of fructose hydrolysis (Nitsch and Nitsch, 1956; Redei, 1974; Nash and Boll, 1975). The overall growth patterns often varied with repeated subculturing, thus making precise recommendations rather difficult.

Whilst sucrose is commonly used for the micropropagation of rose in vitro (Skirvin and Chu, 1979; Davies, 1980; Khosh-Khui and Sink, 1982), it would seem from this study that a glucose+fructose medium gives optimal shoot growth at the sugar concentrations used in these experiments. Indeed, growth on an autoclaved sucrose-medium is presumably reliant on the products of sucrose hydrolysis ie. glucose and fructose, as growth on filter-sterilised sucrose, which contains only sucrose (Thorpe and Meier, 1973), is so poor.

Vitrification remained at a low level for both cultivars during the two experiments, although its development was found to be dependent upon the concentration of sugar present in the culture medium. Shoots grown on a medium containing 10 gl^{-1} of sugar gave rise to 36% of shoots being vitrified, compared with 16% on 20 gl^{-1} sugar. Whilst sugar concentration had a significant effect on vitrification, the type of sugar had no effect, which might be expected, if, as is suggested, the matric or osmotic potential controls the process of vitrification (Debergh et al, 1981).

The two methods of sugar sterilisation resulted in differences in the consistency of the agar medium and in the browning or caramelisation of sugars. Filter-sterilised sugar-media were all firm in texture with no visible signs of discolouration. Autoclaved sugar-media, however, were all less solid, fructose-media in particular being very 'sloppy' with a very obvious brown/amber colouration. Glucose- and sucrose-media also showed visible signs of caramelisation but to a lesser extent than fructose, the browning of sucrose-media being only very slight. In addition, culture jars with autoclaved sugar-media contained large amounts of condensation and excess water was often present on the surface of the medium. These differences in media consistency and amounts of free water in culture jars may well be contributing factors, explaining some of the differences between shoots cultured on the two types of

media.

Twice as many vitrified shoots developed on autoclaved sugar-media (20 gl^{-1}) compared with filter-sterilised sugars. This may well be due to the two factors mentioned previously ie. medium consistency and the presence of free water, both of which are likely to affect the osmotic relations between the plant material and its culture medium. Vitrification has been reduced in several studies by increasing the concentration of agar or sucrose included in the medium (Ziv et al, 1983; von Arnold and Eriksson, 1984), which has been shown to be due to the matric potential, for Cynara scolymus at least (Debergh et al, 1981). Other reports indicate that shoot cultures grown on liquid medium show an increased frequency of vitrification compared with similar cultures on medium solidified with agar (Rugini et al, 1985). Paques (1985) suggests that this difference may be due to hydrolysis of agar in solid medium promoting proliferation and avoiding vitrification. The water relations between shoot material and culture medium may also be important however, especially as in this study with rose, the agar in all the different treatments had undergone hydrolysis during autoclaving.

The increased amounts of surface water and condensation in culture jars may promote excess water uptake and result in increased water contents as seen in vitrified shoots, so encouraging the process of vitrification. The 'sloppy' texture of autoclaved sugar-

media may result in a similar increase in the water content of cultured shoots. This may also affect the relative humidity within the culture vessel, another factor which is reported to control vitrification (Ziv et al, 1983). Reducing the RH, eg. by using a desiccant, is a key factor in preventing the vitrification of plant material, and minimising the accumulation of condensation within culture jars, by increasing the distance between jar and light source, has also proved beneficial (Debergh - private communication).

The results from both experiments indicate that the rate of shoot multiplication may be significantly increased by culturing shoots on alternative sources of organic carbon ie. sugars. Glucose-containing media gave maximal rates of multiplication, averaging 3.2 over the whole study. Other glucose- and fructose-containing media may also improve shoot proliferation and should be considered for improving the growth of rose shoots in vitro.

Fresh weight increase similarly indicated glucose- and fructose-containing media to provide for better shoot growth, although here the method of sugar sterilisation also had an influencing effect. Shoots grown on autoclaved-sugar media had greater fresh weight increases, this again perhaps being linked to the consistency of the medium and excess amounts of water in culture jars increasing the water content of shoots.

It must also be remembered that whilst an equal concentration of each sugar was included into the medium, in terms of gl^{-1} (or w/v), the sucrose will be present at half the osmotic strength (58.428 mM $\equiv$ 20 gl^{-1}) compared with the glucose, fructose and glucose+fructose (111.012 mM $\equiv$ 20 gl^{-1}). This could have its own independent influence on shoot growth and water contents and correspondingly affect the fresh weight data. Even so, the improvement in shoot proliferation by a particular sugar is more likely to be dependent upon its nutritional qualities as opposed to its osmotic properties, and a glucose-medium must therefore be of potential use in the improvement of rose shoot growth in culture.

As well as improving the overall growth of shoots in vitro, the use of alternative sugars may also affect some physiological aspect of shoot growth. For both cultivars, the inclusion of maltose into the medium gave rise to shoots with significantly increased rates of CO_2 uptake. However, the delitirious effect of this sugar on shoot growth must mean that its use is clearly limited. Shoots grown on fructose-media also showed increased photosynthetic rates, although this was not supported by data from the second experiment, where there was no effect of sugar type on the CO_2 uptake of Iceberg shoots. For shoots of Peace however, the same experiment showed a sucrose-containing medium to support very poor growth, and consequently such shoots had very low rates of CO_2 uptake in comparison with shoots grown on the other

sugars.

The effect of sugar type on the chlorophyll content of shoots gave varied results between the two experiments. Whilst FW data from the first experiment for Iceberg indicated maltose to significantly reduce chlorophyll content, the second showed a fructose-medium to significantly increase shoot chlorophyll content. For Peace, the second experiment showed all other sugars to give significantly increased concentrations of chlorophyll compared with a sucrose-medium. The chlorophyll a:b ratios may also reflect abnormal shoot growth, such as that characteristic of maltose-media; increased to 3.77 for Iceberg and decreased to 2.46 for shoots of Peace.

Thus for Peace in particular, sucrose seems to be inadequate in supporting lush, healthy growth, and other sugars should be considered for shoot culture. Even for Iceberg, shoot proliferation may be further improved by the use of another sugar ie. glucose. The sterilisation of sugars is also of interest in the regulation of shoot growth and development. The hydrolysis of media constituents on autoclaving should not be ignored when interpreting results, and its products, notably those of autoclaved sugars, may be important factors regulating the growth and proliferation of rose shoots <u>in vitro</u>.

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CHAPTER 5.

The influence of osmotic potential on the greening of etiolated cotyledons, and on the growth and development of rose shoots *in vitro*.

5.1 ABSTRACT

The effect of increasing the concentration of sucrose or mannitol in order to reduce the osmotic potential, was to significantly decrease chlorophyll development in etiolated cotyledons of radish. The effect of sucrose however, was much more severe, at high concentrations, than that of mannitol, suggesting sucrose to be acting in another role, in addition to any osmotic factors.

The addition of mannitol to culture media containing low concentrations of sucrose may help to reduce the development of vitrified rose shoots, at least for cv. Iceberg, although the percentage of vitreous shoots was low for both cultivars and did not pose any problems. Mannitol had no significant effect on shoot development, and could not replace the sucrose requirement for growth, showing sucrose to have an important nutritional role in the support of shoot growth.

5.2 INTRODUCTION

The osmotic potential (OP) of the culture medium has an important influence on both the growth and development of plant tissue cultures. The water potential is defined as the potential of the water present in a matrix eg. a colloid or soil, and is the sum of three separate components, pressure potential (U_p), osmotic potential (U_π) and matric potential (U_m). The pressure potential (turgor or wall pressure) includes that acting against the force of gravity, whilst solute or osmotic potential results from the salts present in solution. The matric potential is an expression of the various chemical and physical attractions between water and cell walls, organic molecules, soil particles etc. (ie. water adsorption) and results in the retention of water within the soil or culture medium. All three components are closely interlinked and may be altered with corresponding changes in the water relations for the growing plant (Wilkins, 1985).

The incidence of vitrification (hyper-hydrated or water-soaked shoots) is related to the matric or water potential of the culture medium (Debergh et al, 1981). Decreasing the water potential, by an increase in agar or sucrose concentration, reduces the percentage of vitrified shoots which develop, presumably by preventing the excessive water uptake characteristic of such vitreous material. The whole area of vitrification is discussed in detail in Chapter 7.

The water potential of pure free water is designated as zero. When a solute is added to free water, the water potential is lowered ie. becomes more negative as free energy decreases. Osmotic potentials may be reduced by the addition of sugars such as sucrose or glucose, although as these compounds are readily utilised by plant tissue cultures it is preferable to use an alternative, inert osmoticum. Sorbitol is often used for such purposes, however shoot cultures of Malus are reported to show maximal shoot production and optimal fresh and dry weights on sorbitol-containing media compared with glucose, sucrose or fructose (Pua and Chong, 1984). Therefore sorbitol may not be the ideal osmotic agent for some species in culture. Metabolically inactive or inert compounds such as polyethyleneglycol (PEG), polyethyleneoxide (PEO) and carbowaxes are commonly used, as they are compounds of high molecular weight, which are not apparently metabolised by growing cultures (Klenovska, 1973).

The growth of plant tissue cultures may be altered in response to changes in the osmotic potential of the medium (Kozai et al, 1986). Both cell and callus cultures show reduced growth rates when media water potential is reduced (Doley and Leyton, 1970; Maretzki et al, 1972), cells of soybean callus becoming smaller and spherical regardless of which osmoticum is used (Kimball et al, 1975). Osmotic stress has also been found to be an important factor in conditioning embryogenic suspension

cultures of Carica, efficiency being doubled by transferring from 0.3M mannitol or 0.18M sodium chloride to media with lower osmolarities (Litz, 1986). The production of shoots from tobacco callus cultures is also under the control of osmotic potential. Optimal growth and shoot production occurs at 30 gl^{-1} sucrose, with a reduction in productivity at both higher and lower sucrose concentrations. At low sucrose, this productivity may be increased by the addition of mannitol to the culture medium to give the same water potential as that of the 30 gl^{-1} sucrose medium (Brown et al, 1979; Brown and Thorpe, 1980). Thus mannitol can partially replace the sucrose requirement for growth, showing part of the tissue carbohydrate to be acting in an osmoregulatory role. Cultures are not however, capable of growth with mannitol as the sole source of carbon (Nickell and Burkholder, 1950; Douglas, 1985), indicating sucrose to have an equally important nutritional function as well.

The following experiments describe the effect of sucrose, mannitol and their combination on chlorophyll development in etiolated cotyledons. As a continuation of work reported in Chapter 3, the effect of maintaining a constant osmotic potential, as sucrose is reduced in the medium, is also studied, in relation to rose shoot growth, photosynthetic ability and chlorophyll content in vitro.

5.3 MATERIALS AND METHODS

5.3.1 Cotyledon studies

To study the effect of osmotic potential on the greening of etiolated cotyledons, seeds of radish var. French Breakfast were germinated on moistened filter papers in the dark at 20°C for 7 days. Whole etiolated seedlings were then put into 5 cm petri dishes on 2 filter papers moistened with 2.5 ml of various solutions. These were left in the light for 3 days, the fresh weight and leaf area of the cotyledons measured and chlorophyll contents then determined by extraction in 80% acetone.

Sucrose solutions

Etiolated seedlings were placed onto filter papers moistened with sucrose solutions in a range of 0–160 gl^{-1} (0–467.426 mM) ie. 0, 5, 10, 20, 30, 40, 60, 80, 100, 120 and 160 gl^{-1} sucrose.

Mannitol solutions

Filter papers were moistened with mannitol solutions at osmotic strengths equivalent to those of the sucrose solutions used previously ie. 0–467.426 mM.

Sucrose + mannitol

The procedure was repeated as before, but using equimolar solutions, obtained by combining sucrose and mannitol in various proportions as shown in Table 13.

Table 13. Table showing the proportion of sucrose and mannitol used for each solution (1-5) to give a total OP of 116.856 mM (≡40 gl^{-1} sucrose) for each.

		Solution number				
		1	2	3	4	5
sucrose	gl^{-1}	0.0	10.0	20.0	30.0	40.0
	mM	0.0	29.214	58.428	87.642	116.856
mannitol	gl^{-1}	21.29	15.97	10.64	5.32	0.0
	mM	116.856	87.642	58.428	29.214	0.0

For each of the 3 treatments a control of distilled water was also set up, and the data analysed using a one-way anovar.

5.3.2 'Decreasing' sucrose ± mannitol :- rose shoot culture

Shoots of rose cvs. Iceberg and Peace were cultured onto a medium of MS, 8 μM BA, 40 gl^{-1} sucrose and 6 gl^{-1} 'lab m' agar, prepared and autoclaved as previously specified. One third of these shoots were maintained on 'constant' 40 gl^{-1} sucrose for the duration of the experiment. Another third were cultured onto media with progressively reduced concentrations of sucrose over successive subcultures (from the initial 40 gl^{-1}). The final third of the shoots were subcultured onto 'decreasing' concentrations of sucrose, but here mannitol was added to the medium to maintain the OP constant at 116.856 mM, that is equivalent to the initial 40 gl^{-1}

Table 14. Table showing the 3 types of media used for rose shoot culture, 'constant' sucrose or 'decreasing' sucrose ± mannitol to maintain a constant OP (116.856 mM), over 1-6 successive subcultures.

		Subculture number					
		1	2	3	4	5	6
'constant' sucrose	gl^{-1}	40	40	40	40	40	40
'decreasing' sucrose no mannitol	gl^{-1}	/	20	10	5	2.5	0
	mM	/	58.248	29.214	14.607	7.303	0.0
+ mannitol to give 116.856 mM	gl^{-1}	/	10.64	15.97	18.63	19.96	21.29

sucrose. These 3 treatments are shown in Table 14.

Shoots were assessed for CO_2 uptake (IRGA) and chlorophyll contents at the time of each subculture. Measurements of vitrification, shoot multiplication rate and fresh weight increase were also made. Six samples were taken at the end of each subculture period for each treatment. An overall 2-way anovar was then made using all the data.

5.4 RESULTS

5.4.1 Cotyledon studies

From the data, it is clear that sucrose has a direct

effect on chlorophyll synthesis, independent of its osmotic properties. As chlorophyll content expressed on either a fresh weight or leaf area basis showed identical patterns, only the FW data is presented here.

High concentrations of sucrose (above 20 gl^{-1} ≡ 58.428 mM) inhibited the synthesis of chlorophyll in etiolated cotyledons, anything above 40 gl^{-1} (116.856 mM) causing a 50% inhibition compared with controls ie. water. At a concentration of 160 gl^{-1} (467.426 mM), cotyledons contained only a quarter of the chlorophyll present in control material (Fig. 13).

With mannitol replacing sucrose to give equivalent osmotic strengths, decreased osmotic potentials did not cause the same drastic suppression of chlorophyll synthesis, and there was only a gradual inhibition with decreasing OP. The maximum concentration of mannitol used (467.426 mM) only caused a 29.5% inhibition compared with controls, as opposed to a 76% inhibition by an equivalent solution of sucrose (Fig. 13).

When equimolar solutions were employed, with varying combinations of sucrose and mannitol, the degree of chlorophyll suppression was increased, the greater the proportion of sucrose that was present in solution (Fig. 14).

Also evident with decreasing OP was the development of red anthocyanin pigment. Cotyledons placed in water (controls) possessed very small amounts of anthocyanin,

Fig. 13. The effect of increasing concentrations of sucrose (●) and mannitol (o) (ie. decreasing osmotic potentials) on the greening of etiolated radish cotyledons. Bar represents 5% LSD. (n=6)

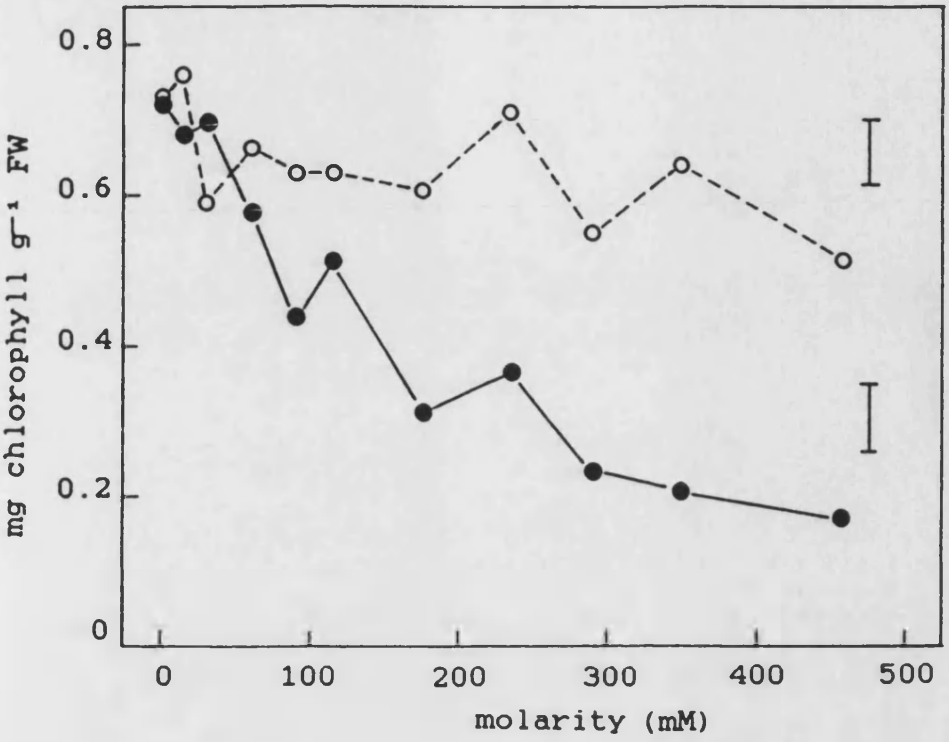
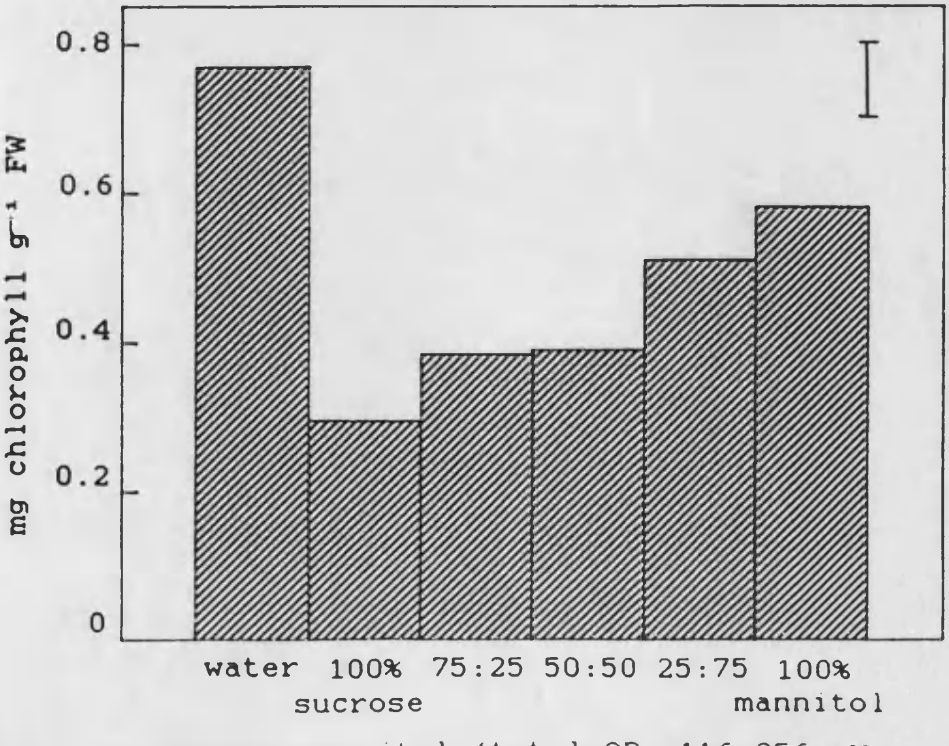


Fig. 14. The effect of equimolar solutions containing various proportions of sucrose and mannitol on the greening of etiolated radish cotyledons. Bar represents 5% LSD. (n=6)



- see Table 13)

this becoming more intense at 58.428 mM (20 gl^{-1} sucrose) and thereafter increasing visibly in concentration with decreasing OP. At 233.713 mM (80 gl^{-1} sucrose, 42.58 gl^{-1} mannitol) the cotyledons appeared totally covered by the red pigment, the anthocyanin masking the green chlorophyll pigments.

5.4.2 'Decreasing' sucrose ± mannitol:- rose shoot culture

Growth characteristics

'Decreasing' sucrose, as shown in Chapter 3, clearly affects both the growth and physiology of rose shoots *in vitro*, although the results here tend to be rather variable. Maintaining the OP constant by the addition of mannitol, may also affect growth, but its precise importance remains unclear from this study.

Whilst levels of vitrification for Iceberg increased as sucrose concentration was reduced, those for Peace remained very low, below 3%, with no significant difference between treatments (Table 15). The addition of mannitol to culture medium with decreasing concentrations of sucrose however, significantly reduced the incidence of vitrification for shoots of Iceberg.

Both shoot multiplication rate and fresh weight increase decreased as sucrose concentration was reduced, even when mannitol was added to maintain the OP constant. The multiplication rate of Peace shoots decreased to about half that of shoots maintained on 'constant' 40

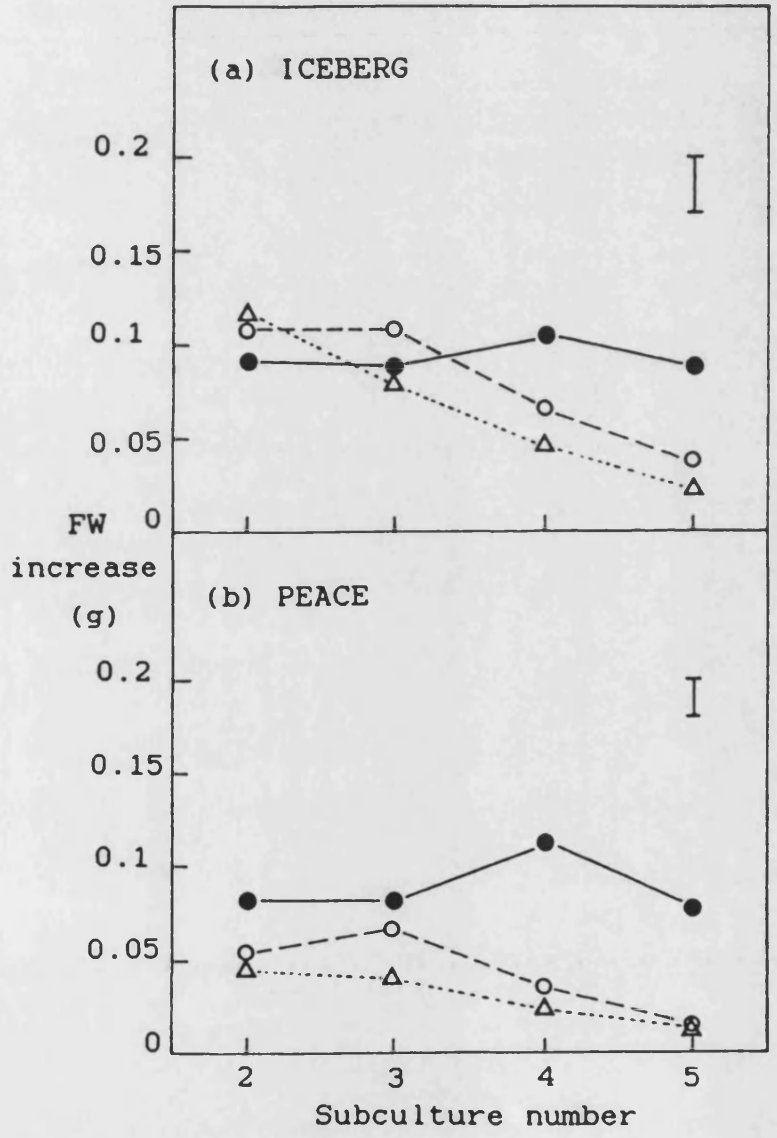
Table 15. The percentage of vitrified rose shoots cvs. (a) Iceberg and (b) Peace developing at each subculture (2)-(5), cultured on media with 'constant' 40 gl^{-1} sucrose and 'decreasing' sucrose ± mannitol.

Concentration of sucrose (gl^{-1}) for 'decreasing' treatment	'Constant' 40 gl^{-1}	'Decreasing' - mannitol	'Decreasing' + mannitol	Mean
(a) ICEBERG				
20 (2)	0.0	8.33	0.0	2.77
10 (3)	0.0	11.25	4.17	5.14
5 (4)	0.0	14.58	0.0	4.86
2.5 (5)	0.0	0.0	0.0	0.0
				(n=6) NSD
Mean	0.0	8.54	1.04	
	5% LSD =4.27 (n=8)			
(b) PEACE				
20 (2)	2.38	1.56	0.0	1.31
10 (3)	0.0	2.38	2.08	1.49
5 (4)	0.0	0.0	0.0	0.0
2.5 (5)	0.0	0.0	5.0	0.0
				NSD
Mean	0.59	0.99	1.77	NSD

Table 16. The rate of rose shoot proliferation cvs. (a) Iceberg and (b) Peace assessed at each subculture (2)-(4), for shoots cultured on media with 'constant' 40 gl^{-1} sucrose and 'decreasing' sucrose ± mannitol.

Concentration of sucrose (gl^{-1}) for 'decreasing' treatment	'Constant' 40 gl^{-1}	'Decreasing' - mannitol	'Decreasing' + mannitol	Mean
(a) ICEBERG				
20 (2)	2.085	2.077	1.902	2.021
10 (3)	2.60	1.825	1.90	2.108
5 (4)	1.50	1.40	1.60	1.50
				(n=6) NSD
Mean	2.062	1.767	1.801	(n=6) NSD
(b) PEACE				
20 (2)	2.716	1.624	1.831	2.057
10 (3)	2.935	1.550	1.757	2.080
5 (4)	2.541	1.30	1.385	1.741
				NSD
Mean	2.731	1.491	1.658	
	5% LSD =0.773			

Fig. 15. The fresh weight increase of rose shoot cultures cvs. (a) Iceberg and (b) Peace grown on 'constant' 40 gl^{-1} sucrose (●) and 'decreasing' sucrose, + mannitol (Δ) or - mannitol (o). Bar represents 5% LSD. (n=5)



gl^{-1} sucrose, being ~ 1.5 compared with 2.7 (Table 16, Fig. 15). The overall pattern of shoot growth and vigour was also influenced by the reduction in media sucrose concentration. Both cvs. exhibited a decrease in shoot vigour with decreasing sucrose, with much slower rates of growth and reduced chlorophyll contents. As has been found previously, this was much more obvious with shoot cultures of Peace than those of Iceberg, the latter cv. being better able to adapt to the gradual reduction in media sucrose.

Several shoots of Iceberg were transferred to a sucrose-free medium, from the 'decreasing' sucrose treatments, at the end of the study. These shoots remained green and healthy, albeit rather small, for 8 weeks, before chlorophyll contents declined and shoots became chlorotic with minimal growth. Due to the fact that the growth of Peace shoots declined more rapidly, none were capable of being transferred to sucrose-free medium.

Physiological characteristics

Shoots of Iceberg showed a significant increase in CO_2 uptake as sucrose concentration was reduced, this being enhanced at 20 gl^{-1} by the addition of mannitol. This improvement in CO_2 uptake was maintained even when the sucrose concentration had been reduced to 2.5 gl^{-1}, although the maximum rate was obtained at 20 gl^{-1} sucrose. Shoots of Peace however, showed no significant

Fig. 16. An assessment of the physiological development of rose shoots cv. Iceberg cultured on media containing 'constant' 40 gl^{-1} sucrose (●) and 'decreasing' sucrose, + mannitol (Δ) or - mannitol (o). Bar represents 5% LSD. (n=6)

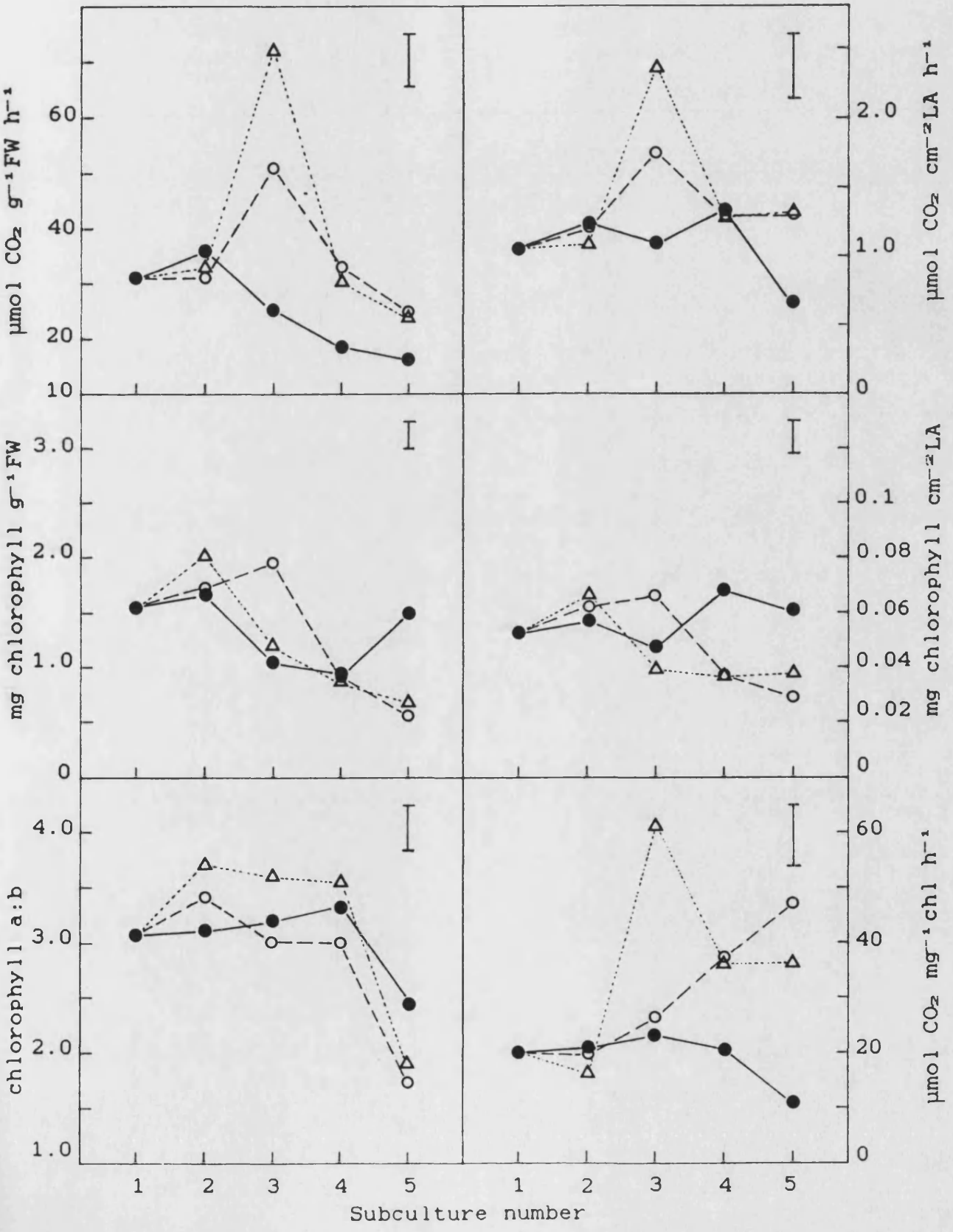
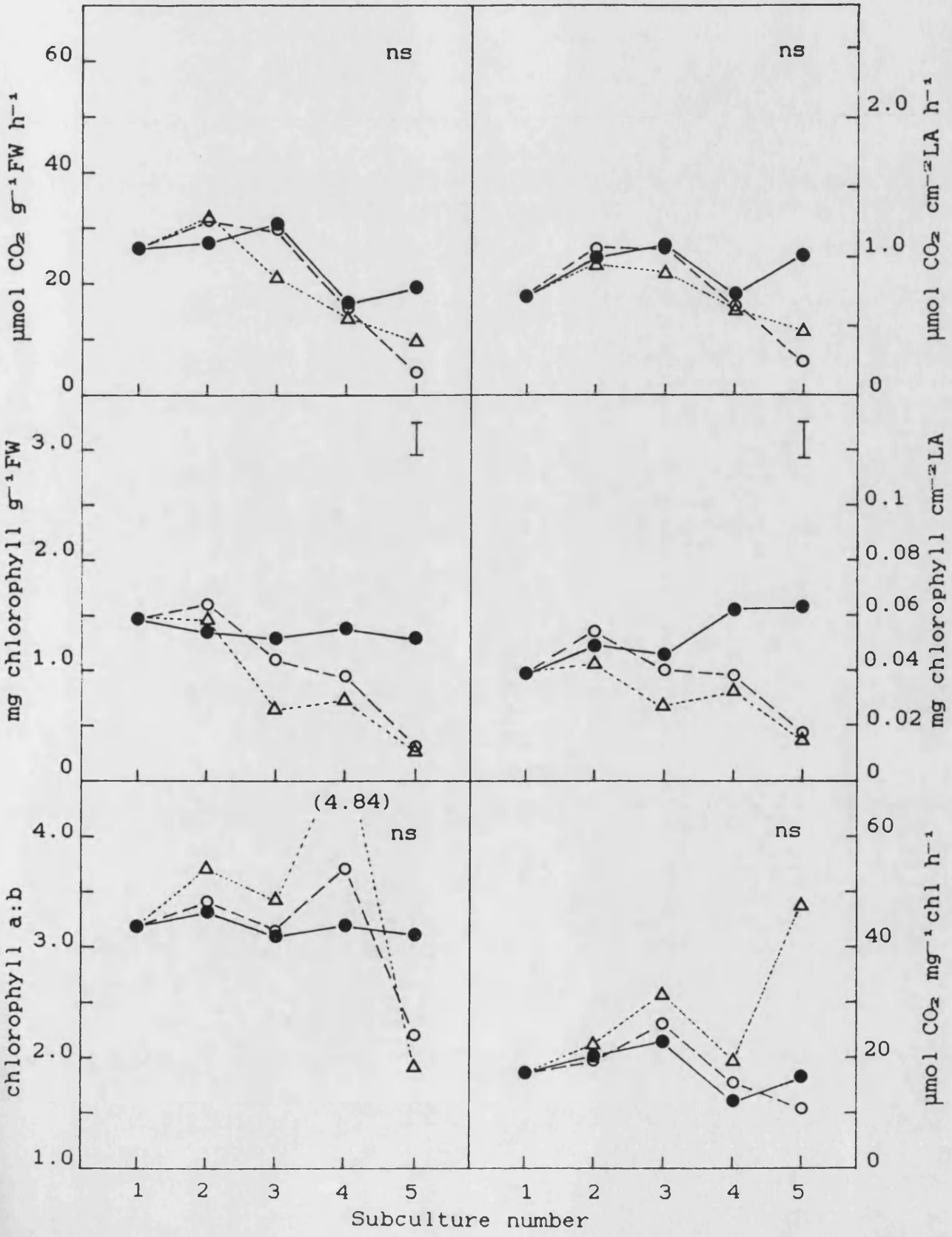


Fig. 17. An assessment of the physiological development of rose shoots cv. Peace cultured on media containing 'constant' 40 gl^{-1} sucrose (●) and 'decreasing' sucrose, + mannitol (Δ) or - mannitol (o). Bar represents 5% LSD. (n=6)



increase or decrease in CO_2 uptake as sucrose concentration was reduced, although below 20 gl^{-1}, rates were decreased with respect to those of shoots on 'constant' 40 gl^{-1} (Figs. 16 and 17).

Chlorophyll contents for shoots of Iceberg increased significantly as sucrose was reduced until the concentration had been dropped to below 10 gl^{-1}, which resulted in a rapid decline. Shoots of Peace also showed a slight initial increase in chlorophyll content as sucrose was reduced, although this increase was not significant, and declined rapidly as medium sucrose was reduced to 10 gl^{-1} and less. For both cultivars, the chlorophyll content of shoots cultured on 'decreasing' sucrose supplemented with mannitol declined more rapidly than those on 'decreasing' sucrose alone (Figs. 16 and 17).

Shoots cultured on a 'decreasing' sucrose medium plus mannitol also had a higher chlorophyll a:b ratio than those on 'constant' 40 gl^{-1} sucrose, this again being characteristic of both cvs. Shoots on 'constant' 40 gl^{-1} sucrose had an a:b ratio of ~ 3.0 for Iceberg and ~ 3.2 for Peace. As sucrose was reduced to 2.5 gl^{-1} however, the a:b ratio of shoots both with and without mannitol dropped to ~ 2.0. The CO_2 uptake per unit chlorophyll increased as sucrose was reduced for both 'decreasing' sucrose treatments $\pm$ mannitol, although for shoots of Peace this increase was not significant (Figs. 16 and 17).

5.5 DISCUSSION

The use of mannitol as an osmoticum can prove useful in the study of sucrose and its osmotic properties. The results indicate that whilst sucrose does have an osmoregulatory role in the growth of shoot cultures, it also has a more important nutritive function as well. This is shown by the fact that mannitol cannot totally replace sucrose in supporting shoot growth (Brown et al, 1979; Brown and Thorpe, 1980; Douglas, 1985).

The cotyledon studies showed sucrose to have a direct effect on chlorophyll development in etiolated tissues, chlorophyll contents decreasing linearly as the concentration of sucrose increased. Equivalent molar concentrations of mannitol also suppressed chlorophyll development, but to a lesser extent than sucrose. An OP of 29.214-58.428 mM (10-20 gl^{-1} sucrose) seemed to be a limiting one, above which chlorophyll development was suppressed.

This suggests that whilst sucrose has an osmoregulatory effect on chlorophyll development, it also has a much more drastic effect on its synthesis, independent of any osmotic properties. This is further supported by the use of equimolar solutions made up of varying proportions of sucrose and mannitol. The greater the proportion of sucrose present in solution, the greater the suppression of chlorophyll synthesis. The nutritional role of sucrose may be the factor responsible

for this increased suppression, altering both the number and morphology of chloroplasts. High concentrations of sucrose may disrupt the internal chloroplast structures and prevent the development of undifferentiated plastids into chloroplasts (Edelman and Hanson, 1971; Pamplin and Chapman, 1975). The development of large amounts of anthocyanin pigment observed as the OP was decreased, may also indicate a stress reaction at high concentrations of sucrose and mannitol.

The addition of mannitol to the medium used for rose shoot culture gave inconclusive results, for both growth and physiological characteristics. The growth of shoots of both cultivars deteriorated as the concentration of sucrose was reduced in the medium, although Iceberg remained more healthy and chlorophyllous at the lower concentrations than Peace. Whilst levels of vitrification were low for both cultivars, the addition of mannitol to the culture medium significantly reduced the percentage of vitrified shoots of Iceberg which developed, compared with the 'decreasing' sucrose treatment lacking mannitol supplements. This suggests vitrification to be influenced, at least in part, by osmotic factors (Debergh et al, 1981).

This decrease in shoot growth observed on sucrose reduction was also reflected in the rate of shoot proliferation and fresh weight increase of cultures. Both these factors declined as the concentration of sucrose was reduced, this being the case whether mannitol was

included into the medium or was omitted. Sucrose must therefore, have a nutritional role in the maintenance of shoot growth which mannitol cannot replace.

As with data from the third chapter, the CO_2 uptake of shoots of Iceberg showed a significant increase as the concentration of sucrose was reduced, this being maintained even when sucrose had dropped to below 2.5 gl^{-1}. The rate of CO_2 uptake peaked when shoots were cultured on 20 gl^{-1} sucrose, thereafter decreasing as sucrose was reduced further. This peak was also reflected by shoots grown on 'decreasing' sucrose supplemented with mannitol, the CO_2 uptake reaching nearly 70 umol CO_2 g^{-1} FW h^{-1}, which is nearly equal to that of material in vivo. There is no apparent reason why shoots cultured on a 'decreasing' sucrose medium plus mannitol should show this large increase in CO_2 uptake, and indeed shoots of Peace showed no similar improvement in CO_2 uptake for either 'decreasing' sucrose treatment, ± mannitol.

Both cultivars showed decreased chlorophyll contents at the lower sucrose concentrations. Again for both cvs., the addition of mannitol to the culture medium caused a more rapid decrease in chlorophyll concentration as sucrose was reduced, possibly suggesting osmotic factors to be involved in chlorophyll development. Whilst high concentrations of sucrose lead to decreased chlorophyll contents, low concentrations also have a similar effect on shoot cultures, showing at least some sucrose to be necessary for the maintenance of chlorophyll. This

presumably acts through a nutritional role, as mannitol cannot supplement low concentrations of sucrose and maintain chlorophyll contents in place of sucrose alone.

The few shoots of Iceberg that were transferred to sucrose-free medium, whilst initially remaining healthy and chlorophyllous, cannot have had a positive carbon balance ie. have been photoautotrophic. After 8 weeks under such culture conditions, shoot chlorophyll contents declined and growth was checked. There was insufficient material to transfer any shoots to soil, and indeed those that were left were small, although initially green and healthy, and may have been slow or even unable to acclimatise to *in vivo* conditions simply because of their size.

In conclusion therefore, whilst osmotic factors are important in the growth of cell and callus cultures (Doley and Leyton, 1970; Maretzki *et al*, 1972; Kimball *et al*, 1975), their precise importance for the culture of whole shoots remains unclear. The osmotic role of sucrose is undoubtedly of importance in the determination of chlorophyll contents, although its nutritive function may also be responsible for the reduction in chlorophyll seen at high concentrations of sucrose. The nutritional role of sucrose is also vital for shoot growth however, as cultures cannot survive and flourish without it and the osmoticum mannitol is unable to fulfil the sucrose requirement for growth.

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CHAPTER 6.

The influence of irradiance levels on the growth and photosynthetic ability of rose shoots *in vitro*.

6.1 ABSTRACT

Shoots of Rosa, cvs. Iceberg and Peace, cultured in vitro at 3 levels of irradiance (3, 10 and 20 Wm^{-2} PAR) showed no significant difference in growth, in terms of shoot multiplication rate or fresh weight increase. At 20 Wm^{-2} however, a visible assessment of growth indicated shoot vigour to be clearly reduced, with a high degree of leaf senescence and lower chlorophyll contents.

The photosynthetic ability of shoots of Iceberg was increased at the lower levels of light, although the FW and LA data were somewhat contradictory, the rather variable FW data resulting in no significant difference between treatments. For shoots of Peace however, photosynthetic rates were maximal at 10 Wm^{-2} PAR, this being significant for both FW and LA data ($p=0.001$). Light irradiance also influenced shoot chlorophyll contents, concentrations increasing significantly with decreasing light for all treatments ($p=0.001$).

6.2 INTRODUCTION

Light is essential for the normal growth and development of plant tissue cultures. Both light quantity and quality have a significant effect on plant growth in terms of biomass, pigmentation etc. The dry weight of illuminated cell cultures of Picea glauca is nearly three times that of cells grown in darkness, showing how important light is for culture growth (Norton and White, 1964). Such light-grown cells are also packed full of chloroplasts with abundant chlorophyll, inducing the development of a photosynthetic apparatus under external conditions. Thus light affects both the growth and physiological development of plant tissue cultures.

Dark-grown tissues contain undifferentiated plastids and their development following light irradiation is dependent upon the wavelength of light. Both blue (467nm) and white (full-spectrum) lights induce the formation of fully differentiated plastids with regular grana stacks and large amounts of chlorophyll. Red (660nm) light prevents any greening of tissue (Berger and Bergman, 1967; Kamiya et al, 1981). Green light is intermediate between red and blue in its action on pigmentation (Dubois, 1973).

As well as controlling plastid differentiation, the quality or wavelength of light also affects the growth and development of plant tissue cultures (Ni et al, 1985a; Norton and Norton, 1986). Blue light is again

reported to stimulate, and is indeed essential for callus growth and shoot production (Seibert et al, 1975). Red and yellow (550nm) lights are both inhibitory and green is intermediate between blue and red (Ward and Vance, 1968; Ni et al, 1985b).

The amount of light the cultures receive is another factor controlling plant growth in vitro (Dunston, 1983; Wainwright and Flegmann, 1984). Daylength is an obvious example where different species show optimal growth under specific light regimes (Dubois, 1973), the micropropagation of rose generally employing a 16h daylength. The irradiance of light ie. the amount received at any one time, also affects the physiology and anatomy of plant cultures, possibly acting in a similar way to the response of sun and shade plants (Dunston, 1983; Lee et al, 1985). Sun plants ie. those growing under high light intensities eg. Helianthus annuus, show a high capacity for photosynthesis and have thicker leaves than those of shade plants. In contrast, shade plants eg. Mercurialis perennis are capable of efficient photosynthesis at low light and have chloroplasts rich in chlorophyll (Boardman, 1977).

The irradiance of light is frequently used to control and improve the weaning of tissue cultured plantlets on transfer to soil. Culture at high light for 1 or 2 weeks prior to transfer may greatly enhance their rate of survival (Hasegawa et al, 1973; Murashige, 1974; Burr, 1976; Makins et al, 1977; Yie and Liaw, 1977; Ziv, 1979).

A similar increase has also been found to be beneficial for the rooting and transfer of micropropagated roses (Bressan and Kim, 1980), in terms of root regeneration (% of plants initiating roots and number of roots per shoot) and increased survival rates (Bressan et al, 1981).

The following study reports the effect of culturing rose shoots under different levels of irradiance (Wm^{-2} PAR). The response of chlorophyll content and the photosynthetic ability of shoots is correspondingly measured.

6.3 MATERIALS AND METHODS

Shoots were cultured on a medium of MS, 8 μM BA, 30 gl^{-1} sucrose and 6 gl^{-1} 'lab m' agar. Cultures were proliferated at 3 different light levels (warm-white), 3, 10 and 20 Wm^{-2} PAR, as measured at culture level inside the jar with the lid in place. Culture jars were placed in a large two-shelf incubator, those on the top shelf being at 20 Wm^{-2} PAR, those on the bottom at 10 Wm^{-2} PAR. To reduce the irradiance to 3 Wm^{-2} PAR, half of the bottom culture jars were covered by a neutral density filter, which reduces the quantity but not quality of light. A 16h daylength was employed.

Shoots were multiplied under these conditions for three 4 week culture periods, and were assessed for CO_2 uptake (IRGA) and chlorophyll content. Growth was assessed in terms of vitrification, shoot multiplication rate and fresh weight increase. Six samples were taken at

the end of each subculture period and an overall one-way anovar made on all the data (n=18) at the end of the experiment.

Temperatures, both inside each culture jar and the ambient level, were measured using a Wescor Inc. TH50 thermocouple thermometer over a 4-5 day period, to take into account any temperature differences due to the different light treatments.

6.4 RESULTS

Temperature measurements

An assessment of temperature, both inside the culture jar and its ambient level, indicated significant differences between the 3 light treatments (Table 17). During the light period (16h) the temperature inside culture jars at 20 Wm^{-2} was significantly increased with respect to those at both 10 and 3 Wm^{-2}, by at least 1°C over that at 3 Wm^{-2}. This was despite the circulation of cooled air in an effort to maintain a uniform temperature throughout the growth cabinet. There was no significant difference between the ambient temperatures of the 3 light treatments, or between the internal temperatures of culture jars during the dark period. During the night period however, the ambient temperature at 20 Wm^{-2} was significantly lower than that at 3 Wm^{-2}, with the temperature at 10 Wm^{-2} being intermediate between the two. During both the light and dark periods the internal temperature of culture jars was greater than their

Table 17. Temperature (°C) measured inside and out of culture jars, with the growth cabinet lights both on and off, at 3 levels of irradiance.

Light irradiance (Wm^{-2})	LIGHTS ON		LIGHTS OFF	
	Internal	External	Internal	External
20	23.25	21.90	21.81	20.62
10	22.57	21.67	21.62	20.81
3	22.17	21.50	21.87	21.00
n	10	10	4	4
5% LSD	0.42	NSD	NSD	0.21

ambient level, this difference being increased at the higher light level.

These temperature differences should be taken into account when interpreting results as they may play some role in determining the pattern of shoot growth.

Growth characteristics

The growth of shoot cultures at the lower irradiance of light was visibly superior to that at 20 Wm^{-2}, with increased chlorophyll contents and healthy vigorous foliage. At 20 Wm^{-2} the older leaves became chlorotic and brown and senesced fairly rapidly. Shoot cultures of Iceberg also possessed small amounts of anthocyanin at 20 Wm^{-2}.

The level of irradiance had no significant effect on either vitrification, multiplication rate or the fresh

Table 18. Measurements of vitrification, multiplication rate and fresh weight increase of rose shoot cultures cvs. (a) Iceberg and (b) Peace, grown at 3 levels of irradiance (Wm^{-2}).

Light irradiance (Wm^{-2})	Vitrification (%)	Shoot multiplication	FW increase (g)
(a) ICEBERG			
20	3.67	2.882	0.143
10	2.35	3.360	0.121
3	1.71	3.325	0.118
5% LSD	NSD	NSD	NSD
(b) PEACE			
20	0.00	2.677	0.085
10	0.00	2.976	0.083
3	2.564	2.876	0.074
5% LSD	NSD	NSD	NSD
n	5	4	15

weight increase of shoot cultures of either cultivar (Table 18). The development of vitrified shoots was minimal for all treatments, all being less than 4%. Rates of shoot multiplication were higher for Iceberg, averaging 3.19 over the 3 light treatments compared with an average of 2.84 for shoot cultures of Peace. Fresh weight increases were also greater for Iceberg, averaging 0.127g compared with 0.081g for Peace.

Table 19. An assessment of the physiological development of rose shoots cvs. (a) Iceberg and (b) Peace cultured at 3 levels of irradiance (Wm^{-2}). (n=18)

LI (Wm^{-2})	µmol CO_2 h^{-1}		mg chl		a:b	µmol CO_2 mg^{-1} chl h^{-1}
	g^{-1} FW	cm^{-2} LA	g^{-1} FW	cm^{-2} LA		
(a) ICEBERG						
20	22.72	0.926	1.186	0.0485	3.27	19.22
10	25.10	1.336	1.556	0.0803	3.33	16.57
3	20.45	1.368	1.566	0.1050	3.16	13.46
5% LSD	NSD	0.256	0.163	0.0108	NSD	NSD
(b) PEACE						
20	22.35	1.001	1.177	0.0528	3.21	18.66
10	27.21	1.371	1.475	0.0753	3.23	18.56
3	20.74	1.035	1.593	0.0799	3.12	13.12
5% LSD	3.51	0.185	0.118	0.0096	NSD	2.19

Physiological characteristics

For shoots of Iceberg, the rates of CO_2 uptake on a FW and LA basis were rather contradictory. The FW data gave no significant difference between light treatments, whereas the LA data indicated a significant improvement at 10 and 3 Wm^{-2} compared with 20 Wm^{-2}. Shoots of Peace however, showed optimal rates of CO_2 uptake at 10 Wm^{-2}, the rates at 20 and 3 Wm^{-2} being very similar and significantly less than that at 10 Wm^{-2} (Table 19).

The chlorophyll content of shoots of both cultivars increased with decreasing light irradiance, these

differences being highly significant ($p=0.001$) for both FW and LA data. The chlorophyll a:b ratios for both Iceberg and Peace were significantly less at 3 Wm^{-2} than those at 10 and 20 Wm^{-2}, but these differences did not prove to be significant. The CO_2 uptake per unit chlorophyll was also decreased at the lower light level, this decrease only being significant however, for Peace.

6.5 DISCUSSION

Whilst light irradiance had no significant effect on the rate of shoot growth, there were visible differences in the patterns of shoot growth for the 3 different light treatments. This is supported in a study by Bressan and Kim (1980) who report that the proliferation of Rosa cv. 'Improved Blaze' is near equivalent at 1000 and 3000lux, but that shoot vigour, in terms of colour, leaf expansion and less senescence, is superior at the lower luminous flux density (1000lux). Although this is primarily due to the effect of light, any temperature differences between the light treatments may also be important.

A temperature of 21°C is reported to be optimal for rose shoot culture and proliferation in vitro, but this can be manipulated to improve root initiation and plantlet survival when transferred to soil (Bressan and Kim, 1980; Bressan et al, 1981, 1982). Temperature can clearly therefore, play a significant role in determining culture growth (de Capite, 1955).

In any growth cabinet there will be differences in temperature with the position of the culture vessel within the cabinet, its distance from the light source etc. This variation is generally recognised and has to be accepted, as even with the cooling system employed in this study, small temperature differences will still arise. Whilst in this study, the internal temperature of culture jars maintained at 20 Wm^{-2} was significantly

higher than those of culture jars at 10 or 3 Wm^{-2}, the internal temperatures at all levels of irradiance decreased when the growth cabinet lights were turned off. These variations in both internal and ambient temperatures with the lights turned on and off, make it difficult to interpret the precise importance of temperature. Whilst a difference of 4 or 5°C is likely to have a significant effect on shoot growth, a variation of 1 or 2°C, which is found within the growth cabinet itself anyway, may prove to be insignificant.

In contrast to the experiments with rose, a similar study with Ribes nigrum L. indicates the level of irradiance to have a significant effect on both shoot proliferation and subsequent in vitro rooting (Wainwright and Flegmann, 1984). The proliferation rate (expressed as shoot doubling time) was lowest at 0.9 Wm^{-2}, increasing to a maximum at 18.5 Wm^{-2}. Any further increase in light failed to improve shoot proliferation beyond the rate at 18.5 Wm^{-2}, this level of irradiance possibly being an upper limit. This is further supported by Bressan et al (1982) who report an increase in shoot multiplication with increased light levels, with maximum rates at 148 uE $m^{-2}s^{-1}$. As with the study on rose however, this was accompanied by decreased shoot vigour and stunted senescent growth.

The deleterious effect of increasing light levels on rose shoot vigour had a corresponding effect on the CO_2 uptake of rose shoots. The response of Iceberg was rather

unclear, as the FW and LA data gave contradictory results, FW data giving no significant difference between light treatments. This may be due to fresh weight variations between treatments, although more likely results from the fact that the FW data collected at the third subculture was particularly variable, thus making the overall statistical analysis non-significant.

A study into the effects of quantum flux density (μE $m^{-2}s^{-1}$ PAR) on the photosynthetic and chlorophyll characteristics of seedling and tissue-cultured plantlets of Liquidambar styraciflua L. is reported by Lee et al (1985). The photosynthetic rate and light saturation point of seedling material is positively related to the level of irradiance, both being greater under high quantum flux densities. For leaves of tissue-cultured plantlets however, those developed under medium light (155 μE $m^{-2}s^{-1}$) had maximal photosynthetic rates compared with both high (315 μE $m^{-2}s^{-1}$) and low (50 μE $m^{-2}s^{-1}$) light. This correlates with the data obtained for Peace, which also showed maximal rates of CO_2 uptake under a medium light intensity (10 Wm^{-2}). The decrease at high light may be due to damage of the light-harvesting pigments (Lee et al, 1985), or possibly, as would seem to be the case with rose, due to decreased vigour at high light intensities.

The chlorophyll characteristics of L. styraciflua L. also correlate with those of Rosa, the chlorophyll content of seedlings and tissue-cultured plantlets being

significantly higher in low-light treated plants. This same pattern was observed with rose cvs. Iceberg and Peace, both FW and LA data giving highly significantly increased amounts of chlorophyll at low light. Lee et al (1985) liken these differences, for seedling material at least, to the characteristics of sun and shade plants. Shade plants have larger chloroplasts and higher total chlorophyll contents than sun plants, although differences in chloroplast structure and starch contents may also influence chlorophyll concentrations. Indeed, Dunston (1983) finds no effect of light irradiance on the number of chloroplasts per unit area in shoot cultures of 4 species in vitro, suggesting chloroplast structure, not density to be affected by light.

The influence of high light on chlorophyll pigments has been widely reported in many in vivo studies, this again being discussed in relation to sun and shade leaves. High light has been shown to break down the chlorophyll pigments, and chloroplasts are developed which contain only a few small thylakoids with a very high photosynthetic activity. In contrast, leaves developed under dim light possess chloroplasts with an enlarged thylakoid system and a much lower photosynthetic activity (Grumbach and Lichtenthaler, 1982). Thus the adverse effect of 'high light' on chlorophyll contents (20 Wm^{-2} is relatively high compared with the level of 10 Wm^{-2} normally used for shoot culture), as seen with rose, is not totally unexpected.

This would seem to contradict the studies which report increased light levels to promote rooting and enhance shoot establishment, although this may be accounted for by a difference in light requirement between the two stages. The data presented in this chapter refers to shoot proliferation, and there is no reason to assume that the same results would be obtained during the rooting process (Bressan and Kim, 1980; Bressan et al, 1981, 1982). This will be assessed in greater detail in the final discussion.

As with tissue-cultured material of L. styraciflua L., irradiance had no effect on the chlorophyll a:b ratio of rose shoot cultures in vitro.

Thus, whilst light may be of use in the manipulation of chlorophyll contents and possibly the CO_2 uptake of rose shoots, it has no beneficial effect on shoot growth in terms of multiplication rate or fresh weight increase. The decrease in CO_2 uptake at the higher irradiance level (20 Wm^{-2}) may be due to the decreased vigour and reduced chlorophyll content of both Iceberg and Peace shoots, which was clearly visible at this level of light.

An irradiance of 10 Wm^{-2} is commonly used for the culture of plant material in vitro, any further increase being limited by the type of growth cabinet and light source used. The results from this study indicate that for the in vitro culture of rose at least, 10 Wm^{-2} gives healthy chlorophyllous shoot growth.

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CHAPTER 7.

Vitrification: a general study and discussion.

7.1 ABSTRACT

During this study on Rosa, the concentration of sucrose in the culture medium influenced the development of vitrified shoots, the proportion increasing significantly with decreasing sucrose. Vitrified shoots were induced to develop by the addition of sterile water to culture jars, suggesting both the water potential and the water relations between the growing shoot and the culture medium to be of importance in the regulation of vitrification.

Vitreous leaves had reduced photosynthetic rates and lower chlorophyll contents compared with those of 'normal' healthy shoots. There was a difference however, between FW and LA data, as the excess water contained in vitreous leaves resulted in an exaggeration of the differences between vitreous and healthy material, as shown by the FW data. Vitreous shoots were discarded as they rarely recovered normal growth and were unlikely therefore, to survive the transfer to soil.

7.2 INTRODUCTION

Vitrification is not generally a problem in the micropropagation of roses, although other species such as Dianthus frequently show severe levels of vitrification (Hakkaart and Versluijs, 1983). Leaves of vitreous or hyper-hydrated shoots appear rather brittle and translucent, contain increased amounts of water and reduced concentrations of chlorophyll (Kevers et al, 1984). Internally, vitreous shoots lack a clear differentiation between palisade and spongy mesophyll, contain less lignin and have less well differentiated xylem and sclerenchyma ie. show reduced internal support (Kevers et al, 1984; Vieitez et al, 1985). Preliminary work with Dianthus plantlets in vitro shows that the internal structure of vitreous shoots is similar to that of the submerged leaves of species possessing a land and water form of leaf (Leshem, 1983a), indicated by mesophyll cells with large vacuoles and the presence of fewer stomata.

Several factors are suggested to contribute to the development of vitrified shoots, and one or all of them may be of importance for a particular species. These include amounts of ammoniacal nitrogen free in the medium (Daguin and Letouze, 1985, 1986), levels of ethylene within the culture environment (Kevers et al, 1984; Kevers and Gaspar, 1985), BA concentrations (Hussey, 1977, 1978) and matric and water potentials (Debergh

et al, 1981, Leshem, 1983a). Vitrification has been reduced in several studies by increasing the concentration of agar included in the medium, suggesting it to be under the influence of matric potential (Debergh et al, 1981; Ziv et al, 1981; Leshem, 1983a, b; Ziv et al, 1983; von Arnold and Eriksson, 1984; Pasqualetto et al, 1986). Other reports indicate that cultures grown on liquid medium show increased levels of vitrification compared with culture on a solid medium (Rugini et al, 1985). This may be due to the hydrolysis of agar in solid medium promoting the development of healthy shoots (Paques, 1985), although the water relations between the shoot and its culture medium may also encourage the process of vitrification in liquid medium.

Whilst the reduction in vitrification resulting from an increased concentration of agar may be due to factors associated with the matric potential, it has also been suggested that increased agar reduces the availability of cytokinins. This would support the idea that under conditions of high matric potential (or low agar), cytokinins enhance the vitrification process (Debergh et al, 1981; Bornman and Vogelman, 1984; Pasqualetto et al, 1986). The role of BA may also be reflected in the ability of activated charcoal to enhance shoot recovery, presumably by the withdrawal of BA from the medium (Constantine, 1983). These two factors and indeed any of the others, may well be interrelated, all contributing in some way if conditions are favourable.

The high level of relative humidity within the culture vessel, which generally approaches 100%, is likely to enhance the excessive amounts of water uptake characteristic of vitreous material. Reducing the RH in culture vessels by the use of a desiccant or by using a looser type of closure (eg. cotton wool) both decrease levels of vitrification (Hakkaart and Versluijs, 1983; Ziv et al, 1983). This is beneficial in commercial terms as vitreous plants do not survive the transfer to soil if the process of vitrification is well established. The leaves of such plantlets lose too much water and soon perish, and are particularly susceptible to infection (von Arnold and Eriksson, 1984).

A general study was made therefore on the photosynthetic and chlorophyll characteristics of vitreous shoot cultures. Data from other chapters is brought into the discussion so that an overall account of the development and physiological features of vitrified shoot material can be included.

7.3 MATERIALS AND METHODS

Vitrified rose shoots cvs. Iceberg and Peace were assessed for CO_2 uptake (IRGA) and chlorophyll during any experiment where they developed in sufficiently large numbers to be significantly analysed.

An attempt to encourage or induce the development of vitrified shoots in vitro was also made. Shoots were cultured on a medium of MS, 8 μM BA, 20 gl^{-1} sucrose and

6 gl^{-1} 'lab m' agar. Shoots were grown on this medium with or without the addition of 5ml sterile distilled water, those cultured without added water being used as controls. Both vitreous and healthy (control) shoots were assessed for their growth and physiology.

7.4 RESULTS

Vitreous shoots of rose were characterised by having enlarged, water-soaked leaves, which were brittle and translucent in appearance. They had greatly increased fresh weights, as twice as many healthy leaves were obtained for an equivalent fresh weight of vitreous material.

A comparison of healthy and vitreous shoots of Peace developed on a multiplication medium with 20 gl^{-1} sucrose during the course of an experiment, indicated clear differences between the two types of shoot. Vitreous shoots had reduced photosynthetic rates and lower chlorophyll contents expressed on both a fresh weight and leaf area basis. They also possessed a reduced chlorophyll a:b ratio, although showed no difference in CO_2 uptake per unit of chlorophyll compared with healthy material (Table 20).

The addition of sterile water to culture jars enhanced the development of vitrified shoots. Vitreous shoots of Iceberg produced by such a method showed reduced levels of both CO_2 uptake and chlorophyll content, although the reduction in CO_2 uptake per unit

Table 20. Measurements of 'normal' healthy and vitreous shoots of rose cv. Peace developed on a multiplication medium of 20 gl^{-1} sucrose. (n=6)

Shoot type	µmol CO_2 h^{-1}		mg chlorophyll		a:b	µmol CO_2 mg^{-1}chl h^{-1}
	g^{-1}FW	cm^{-2}LA	g^{-1}FW	cm^{-2}LA		
'Normal' healthy	42.22	1.093	1.822	0.0469	3.25	23.17
Vitreous	10.03	0.496	0.410	0.0194	2.52	25.02
5% LSD	10.77	0.422	0.133	0.0057	0.25	NSD

leaf area was not found to be a significant one. The chlorophyll a:b ratio was not affected by vitrification, whereas CO_2 uptake per unit chlorophyll was greatly increased for vitreous shoots (Table 21). Vitreous shoots of Peace, obtained by the addition of sterile water to culture vessels, again showed a reduction in their CO_2 uptake and chlorophyll content, although only the FW data proved to be significant when compared with healthy material. Both chlorophyll a:b ratio and CO_2 uptake per unit chlorophyll were unaffected by the process of vitrification (Table 21).

Table 21. Measurements of 'normal' healthy (-) and vitreous (+) shoots of rose cvs. Iceberg and Peace developed on a multiplication medium with 20 gl^{-1} sucrose ±the addition of 5ml sterile distilled water. (n=12)

	ICEBERG			PEACE		
	Shoot type					
	Healthy	Vitreous	5% LSD	Healthy	Vitreous	5% LSD
g^{-1}FW	16.75	9.09	2.95	13.88	4.92	5.43
µmol CO_2 h^{-1} cm^{-2}LA	0.685	0.550	NSD	0.509	0.332	NSD
g^{-1}FW	1.173	0.297	0.154	0.685	0.303	0.228
mg chl cm^{-2}LA	0.0481	0.0179	0.0078	0.0253	0.0199	NSD
a:b	2.65	2.64	NSD	2.71	2.97	NSD
µmol CO_2 mg^{-1}chl h^{-1}	15.13	32.10	6.49	24.12	16.63	NSD
% Vitr	0.13	28.86	p=0.01 (t-test)	29.36	50.65	p=0.05 (t-test)

7.5 DISCUSSION

Vitrification can be a problem in the micropropagation of some species, but in none of the studies with Rosa did vitrified shoots develop in sufficiently large numbers so as to severely affect their culture. Vitrified (or hyper-hydrated) shoots are undesirable as they rarely recover 'normal' growth and therefore have to be discarded (Leshem, 1983b; Ziv et al, 1983). Some recovery may be possible however, if the process of vitrification is not too advanced and shoots are not too severely affected (Hussey, 1977). Slightly vitrified shoots of Picea abies L. can be acclimatised to greenhouse conditions, although severely affected shoots soon perish (von Arnold and Eriksson, 1984). Further studies with Dianthus indicate that even when vitreous shoots are recovered, rooted and grown on, there may be some disruption of chimaeral structure, with the development of colour variation. This could be of commercial interest in the case of Dianthus, but for other species may be highly undesirable (Leshem, 1986).

Studies with Rosa cvs. Iceberg and Peace, show the concentration of sucrose included in the culture medium to be important in controlling vitrification. The percentage of vitrified shoots was significantly increased at low sucrose (10 gl^{-1}), this proportion increasing as sucrose was decreased in the medium over successive subcultures. Maene and Debergh (1985) find similar results, also with rose, where raising the

sucrose from 30 to 40 gl^{-1} reduced the degree of vitrification. A concentration of 30 gl^{-1} sucrose or higher is commonly used for rose micropropagation, the culture of miniatures using 45 gl^{-1}, as problems with vitrification were encountered at lower concentrations (Anon, 1982).

The addition of mannitol to supplement low concentrations of medium sucrose decreased the number of vitrified rose shoots cv. Iceberg which developed, although similar studies with Malus and Dianthus did not prevent abnormal leaf development (Constantine, 1983; Ziv et al, 1983).

The precise factors controlling the process of vitrification remain unclear, although there may be differences between species dependent on the culture conditions used etc. The vitrification of almond shoot cultures is reported to be independent of BAP concentration, pH and osmolarity of the medium, but dependent upon carbon source and both the quality and quantity of gelling agent used (Rugini et al, 1985). Whilst the vitrification of rose was not influenced by carbon source, the use of 45 gl^{-1} fructose for almond drastically decreased vitrification compared with 51 gl^{-1} sucrose or sorbitol.

There is also no one factor involved in the vitrification of Malus (Constantine, 1983). In only two experiments was it found to be related to the culture

medium; where there was a large increase in the concentration of cytokinin or sucrose on transfer to fresh medium. Attempts to reduce the development of vitrified shoots by reducing the osmotic potential of the culture medium, with the addition of mannitol or agar, also proved unsuccessful.

Vitreous shoots do not generally proliferate (Constantine, 1983) and contain reduced amounts of chlorophyll, giving rise to their pale, translucent appearance (Phan and Letouze, 1983; Kevers et al, 1984). Preliminary work with Dianthus in vitro also shows that whilst photosynthesis does occur in vitreous shoots, it is reduced to about half the rate of CO_2 uptake in normal healthy shoots (Leshem, 1983a). Vitreous shoots of rose cv. Peace, developed on 20 gl^{-1} sucrose during the course of an experiment, showed significantly reduced rates of CO_2 uptake, decreased chlorophyll contents and a lower chlorophyll a:b ratio, when expressed on a FW or LA basis, compared with healthy material.

It must be remembered however, that when CO_2 uptake or chlorophyll content is assessed per g FW, any differences between vitreous and healthy material will be exaggerated due to the increased water content of vitreous leaves. The difference between FW and LA data was clearly demonstrated by the figures for Peace. The CO_2 uptake and chlorophyll content of shoots was reduced to around 25% and 50% of those of healthy shoots, on a FW and LA basis respectively. The FW data exaggerates the

difference between the 2 types of shoot, as healthy shoots will have a higher dry matter content and a greater number of leaves than an equivalent fresh weight of vitreous material, giving a greater uptake of CO_2.

This difference is evident when the data for Iceberg and Peace with or without the addition of sterile water to culture jars is considered. Whilst vitreous shoots have reduced rates of CO_2 uptake for both FW and LA data, only the FW data proved to be significant. Similarly for chlorophyll contents, the reduction for shoots of Peace was only significant for the FW data, although both the FW and LA data were significant for Iceberg. In contrast with the first study on Peace, the second study indicated the chlorophyll a:b ratio to be unaffected by vitrification for both cvs., although it was rather low, around 2.65 for Iceberg and 2.83 for Peace.

Whilst the CO_2 uptake per unit chlorophyll was unaffacted by vitrification for Peace, vitreous shoots of Iceberg had significantly increased photosynthetic rates per unit chlorophyll. This may be due to healthy shoots of Peace having low chlorophyll contents in comparison with Iceberg so that the decrease in chlorophyll in vitreous shoots of Iceberg is exaggerated when compared with Peace. This large difference in chlorophyll content between healthy and vitreous material of Iceberg results in the CO_2 uptake per unit chlorophyll being much less for healthy than vitreous material.

Whilst the majority, if not all, reports concerning the vitrification of plant material in vitro indicate it to be an undesirable characteristic, it has been shown to be greatly beneficial for the production of sitka spruce shoots (John, 1986). Vitrified shoots were induced to develop by submergence, sterile water being added to culture vessels to a depth of 10 or 30 mm following initial establishment procedures. The submergence treatment induced vitrification in all cultures and significantly promoted shoot production, the meristems reverting to a normal pattern of growth after emergence from the water. John (1986) suggests therefore that vitrification may be a useful phenomenon in the micropropagation of sitka spruce and should be actively encouraged. A scheme whereby shoot vitrification is induced by submergence is proposed, followed by water removal to allow vitreous growth to normalise. Normal cultures give rise to 3.3 apices compared with 9.2 and 5.9 for vitreous and reverted shoots respectively.

In conclusion therefore, whilst the development of vitreous shoots is generally undesirable, it is not usually a problem in the micropropagation of rose. As they contain reduced concentrations of chlorophyll and low rates of photosynthesis, together with an abnormal internal structure, they rarely survive the transfer to soil conditions, and unless are capable of reversion to normal growth, have to be discarded. In cases where vitrification is a problem, severely reducing the number

of healthy plantlets produced, efforts to reduce its development by decreasing the water potential of the culture medium or reducing the concentration of BAP etc. may prove useful.

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CHAPTER 8.

A study into the rooting of rose shoots, both *in vitro* and *in vivo*, and the influence of varying concentrations of media sucrose prior to the rooting period.

8.1 ABSTRACT

Cultured shoots of Rosa cvs. Iceberg, Peace and Fragrant Cloud were used to study the growth and physiological development of both the in vitro foliage and the foliage developed within the in vivo environment on transfer to soil. Shoots were either rooted in vitro, with the use of 1/2 MS, 0.1 mg l^{-1} NAA and 30 gl^{-1} sucrose, or in vivo (in soil) with the direct transfer of shoots following an auxin-powder dip. Prior to both these treatments, shoots were cultured on a hormone-free 'elongation' medium, with the inclusion of activated charcoal to promote shoot vigour and encourage the development of well-expanded healthy foliage.

Shoots did not root well in vitro, with a loss of chlorophyll pigment and an overall decline in shoot vigour. An in vivo rooting system was therefore employed. The influence of decreased concentrations of media sucrose during the pre-transfer period indicated no significant effect on the subsequent rooting and establishment of shoots. The foliage developed under soil conditions (the in vivo leaves) had significantly increased rates of photosynthesis, with increased chlorophyll contents and, more importantly, a positive carbon balance compared with the foliage retained from culture. The in vitro foliage however, achieved its maximal rate of CO_2 uptake and a small degree of photosynthetic competence after 7-14d under soil conditions, at which time the emergence of the in vivo

foliage became apparent.

The foliage retained from culture may therefore, make a significant contribution to the growth of the establishing plantlet, providing it with nutrients accumulated during the previous pre-transfer culture period. After the nutrients and minerals have been withdrawn from the in vitro leaves, they begin to degenerate and senesce, with a rapid decline in photosynthetic rate, chlorophyll content and level of photosynthetic competence.

8.2 INTRODUCTION

The rooting and establishment of tissue cultured plantlets is the final stage in the process of micropropagation, and is of vital importance in producing healthy, quality material. If conditions do not provide for optimal growth and acclimatisation, large numbers may be lost, thus increasing the price of the remaining plantlets. Whilst many species are induced to form roots *in vitro*, it is generally accepted that *in vivo* rooting, if possible, is preferable, for a number of important reasons (Debergh and Maene, 1981; Conner and Thomas, 1982; Maene and Debergh, 1983):

(1) *In vitro* rooting is very labour intensive, accounting for at least 35% of the total cost. Single shoots have to be excised and handled individually (Anderson, 1978; Donnan *et al*, 1978).

(2) It is difficult to induce a good functional root system *in vitro*, as there is usually little branching, a lack of root hairs etc., and in most cases the *in vivo* roots die off after 2 weeks in soil, with the development of new roots *in vivo*. This results in a delay in shoot growth, which may correspondingly affect plantlet quality and prolong the establishment process. There may also be incomplete vascular connections between root and shoot, if rooting *in vitro*, causing problems in the control of water loss, water transport etc. (Grout and Aston, 1978; Sutter, 1982).

(3) It may be difficult to obtain optimal rooting

in vitro, as exogenous auxins are needed for initiation, but not for elongation. As shoots are kept in constant contact with an auxin-containing medium for up to 2 weeks, it will not be possible to provide conditions favourable for these two rooting factors. This can be overcome by culture on an auxin-medium for a few days, followed by transfer to hormone-free (James and Thurbon, 1979).

(4) The roots developed *in vitro* may be damaged when the rooted shoot is transferred to soil, increasing the opportunity for root and/or stem disease.

The alternative of rooting shoots *in vivo* may be achieved in several ways, the cultured shoot being treated as a minicutting:

(1) The shoot base can be dipped in an auxin solution (possibly with the addition of sucrose, vitamins and minerals) for a few days, or even minutes prior to transfer to soil (Hughes *et al*, 1973; Avramis *et al*, 1982; Douglas, 1984; Samartin *et al*, 1986). Alternatively a hormone rooting powder may be used.

(2) The medium to which the cultured shoots are to be transferred eg. peat, vermiculite, may be saturated with an auxin-solution prior to shoot transfer.

(3) An exhausted medium may be replenished with minerals, sugars etc. by the addition of liquid supplements to culture vessels (Maene and Debergh, 1985). This removes the need to transfer shoots to fresh medium, reducing both the cost and labour involved in the rooting

procedure.

The rooting of rose shoots is generally carried out *in vitro*, although is as equally successful *in vivo* (McCown, 1980), and there would appear to be little advantage in *in vitro* rooting, other than the ability to maintain defined, controlled conditions and to enable direct comparison between various treatments.

The inclusion of auxins into the rooting medium, whilst not essential for root development, both enhances and reduces the length of the rooting process. The type of auxin used may significantly influence rooting, with NAA being recommended for rose in several studies. This auxin enhances root initiation together with an improvement in the successful transfer of shoots to *in vivo* conditions, the latter feature clearly being important in economic terms (Hasegawa, 1980). Khosh-Khui and Sink (1982) report an additive effect of NAA and IAA on rooting, with an improvement in root quality at most concentrations tested.

The majority, if not all reports in the literature show the use of reduced concentrations of mineral salts (1/8 to 1/2 MS) to significantly improve root formation and transplant survival, higher concentrations being inhibitory (Hasegawa, 1980; Short *et al*, 1981; Sauer *et al*, 1985). This has been shown to be due to a reduction in the concentration of nitrogen salts, or an increase in the sucrose:nitrogen ratio, and results in an increase in

both root number and length (Hyndeman et al, 1982a, b).

Another factor influencing the rooting and establishment of cultured shoots is light. Although this has not been studied in this thesis, an increase in the irradiance of light provided during rooting has been shown to increase the initiation of roots and also enhance the successful transfer of rose shoots to soil (Bressan and Kim, 1980; Bressan et al, 1981, 1982). Higher light during the proliferation stage increases the time of root emergence for shoot cultures of Ribes, although has no effect on the final rooting percentage which exceeded 90% for all treatments (Wainwright and Flegmann, 1984). This has been reported in several studies with various species, eg. ferns (Burr, 1976), Ficus (Makins et al, 1977), papaya (Yie and Liaw, 1977), a light increase enhancing not only the rooting process itself, but possibly also increasing rates of CO_2 uptake or 'boosting' the photosynthetic system prior to transfer.

Increasing the light level for shoot cultures of Asparagus results in the differentiation of cladophylls (scale-like 'leaves') ie. the ferning of spears. This is associated with a marked increase in the survival of plants when transferred to soil, possibly due to the cladophylls being more capable of photosynthesis and autotrophic growth than unferned spears (Hasegawa et al, 1973; Murashige, 1974). Daylength may also influence rooting to some extent, although its precise importance

may vary between species (Lanphear and Meahl, 1961; French, 1983).

Several other factors also have potential to improve the rooting process. The addition of activated charcoal may enhance root initiation, with the adsorption of phenols and the removal of any cytokinin remaining within the plant tissues from shoot proliferation (Bressan *et al*, 1981; Snir, 1981). The charcoal also provides for a darker environment which may correspondingly encourage root development. The use of a liquid medium may prove to be beneficial, the improved aeration giving much longer roots than in agar, and enhancing the development of root hairs (Sriskandarajah and Mullins, 1981; Lee *et al*, 1986).

The following study investigates the rooting of rose shoots, both *in vitro* and *in vivo*, with the use of various techniques and media. The manipulation of sucrose concentration prior to transfer to soil is also studied, and the development of *in vivo*, and 'adaptation' of *in vitro* foliage is correspondingly followed. The two main cultivars used are Peace and Fragrant Cloud (both Hybrid-Teas), problems being encountered with Iceberg so that insufficient shoots were obtained to complete the whole study.

8.3 MATERIALS AND METHODS

Proliferating shoots, cultured on a medium of MS, 8 µmol BA, 30 gl^{-1} sucrose and 6 gl^{-1} 'lab m' agar, were

used for the rooting studies. The medium used for shoot elongation contained MS, sucrose, 1 gl^{-1} activated charcoal and 6 gl^{-1} 'lab m' agar, with no plant growth regulators. Five shoots were placed in each culture jar.

The rooting medium contained $\frac{1}{2}$MS, sucrose and 0.1 mg l^{-1} NAA, with 6 gl^{-1} 'lab m' agar for solid medium, and lacking agar for liquid medium (5 shoots per jar). During all <u>in vitro</u> work, culture jars were maintained in growth cabinets at 20°C, 10 Wm^{-2} and with a 16h day.

The <u>in vivo</u> rooting studies involved the transfer of cultured shoots direct from the elongation medium to soil. The base of each shoot was washed free of any remaining agar, dipped into Seradix No. 1 rooting powder (for softwood cuttings, active ingredient 4-indol-3-ylbutyric acid), the excess tapped off and the shoot then placed into a peat/perlite mix (50/50) in seed trays, with plastic covers to maintain humidity. The trays were maintained in a growth cabinet at 20°C day/15°C night, with an irradiance of 10 Wm^{-2} and a 16h day. The soil was kept moist by spraying every 4 or 5 days, being careful to avoid waterlogging. After 7d, the vent in the cover was half opened and after 14d was fully opened. The established plantlets were transferred to individual 3 inch pots after 28d and left to grow on in the greenhouse under normal conditions of daylight and length.

After 7d in soil, the leaves persistent from culture were tagged with cotton so as to enable identification of

the *in vitro* foliage and the recognition of leaves developed during *in vivo* establishment.

Shoots (ie. *in vitro* leaves) were assessed for CO_2 uptake (IRGA) and chlorophyll at the end of each *in vitro* treatment period, and after 7, 14, 28 and, if sufficient material, 56d in soil. The *in vivo* foliage, ie. that developed under soil conditions, was similarly assessed after 14d plus.

8.3.1 *In vitro* rooting

Rose shoots, cvs. Iceberg and Peace, were transferred from the proliferation medium (30 gl^{-1} sucrose) to one for elongation (30 gl^{-1} sucrose). After 14d they were transferred to a rooting medium (30 gl^{-1} sucrose), either solidified with agar, or to culture jars containing 5 sorbarods (Sorbarod Systems, Hull) soaked with 25ml liquid rooting medium. After a further 14d, the shoots were transferred to soil for weaning and establishment.

8.3.2 *In vivo* rooting

Varying sucrose concentrations during shoot elongation

Shoots of cv. Fragrant Cloud were transferred from the proliferation medium (30 gl^{-1} sucrose) to an elongation medium containing 10, 20 or 30 gl^{-1} sucrose. After 14d they were transferred straight to soil, omitting the *in vitro* rooting stage.

Decreasing sucrose concentrations prior to and during shoot elongation

Shoots of cvs. Peace and Fragrant Cloud were proliferated on medium containing 30 gl^{-1} sucrose. At the next subculture, half of the shoots were transferred to a proliferation medium with 20 gl^{-1} sucrose, half to medium with 30 gl^{-1} sucrose. After 4 weeks, ie. one subculture period, the shoots were transferred to an elongation medium, those on 20 gl^{-1} sucrose to a 10 gl^{-1} medium, and those on 30 gl^{-1} maintained on 30 gl^{-1} sucrose. The two treatments are referred to as 'decreasing' and 'constant'. After 14d on elongation medium, the shoots were transferred to soil as before.

8.4 RESULTS

8.4.1 In vitro rooting

No quantitative data is presented on the rooting *in vitro* of rose as the percentage of shoots that rooted was less than 50%. As the primary interest of the experiment was to have a rooting and weaning system that could be used routinely to investigate the photosynthetic ability of shoots, the experimental effort was switched to an *in vivo* rooting/weaning procedure (see 8.4.2). A general description of shoot elongation and *in vitro* rooting is presented.

The use of a charcoal medium for shoot elongation prior to both *in vitro* and *in vivo* rooting, was greatly beneficial (Plates 1, 2 and 3a), with all three cultivars

of rose used in this study developing large, well-expanded healthy foliage. As was the case on proliferation medium, shoots of Peace, although still showing improved shoot growth and leaf expansion, remained more compact, with less shoot extension than shoots of both Iceberg and Fragrant Cloud. If shoots of either 3 cultivars were left on the charcoal medium for more than 14d, their vigour rapidly declined, with the onset of leaf senescence and chlorosis.

Although roots did develop, both in solid and liquid medium, they were long, thick and unbranched, often being brown in colour and with no root hairs. There seemed to be little difference between the 2 types of media used for rooting, although the sorbarods soaked with liquid medium tended to remain rather waterlogged, with a resulting adverse effect on shoot vigour. Shoots of Iceberg and Peace rooted <u>in vitro</u> showed very poor vigour after 14d on both solid and liquid media, the older leaves becoming senescent and chlorotic. This was despite the previously excellent growth of shoots after culture on the charcoal elongation medium (in particular those of Iceberg - Plate 1).

Associated with waterlogged sorbarods was the observation that the roots often grew out along the surface of the sorbarod and then down between the central tissue-like material and its thin plastic covering. The shoot was not therefore, being rooted firmly into the sorbarod. On the contrary, the shoot was often pushed out

of it, so that when it came to transfer the rooted shoot plus sorbarod to soil, the shoot came loose, resulting in root damage. Also, the sorbarods being so water-logged tended to lose their shape and collapse fairly readily when handled, all making the transfer of such shoots *ex vitro* rather difficult and inefficient.

On transfer to soil, the poor vigour of *in vitro*-rooted shoots resulted in very low rates of survival, even the possession of *in vitro* roots proving to be of no apparent benefit. Indeed, it was clear that these roots soon perished in the soil environment, establishment depending on the growth of new roots *in vivo*.

Plate 1. The growth of rose shoots cv. Iceberg on (a) shoot proliferation and (b) shoot elongation media, after a 4 week and 2 week culture period respectively, both containing sucrose at 30 gl^{-1}. (Scale X2.0)

(a) Shoot proliferation

(b) Shoot elongtion

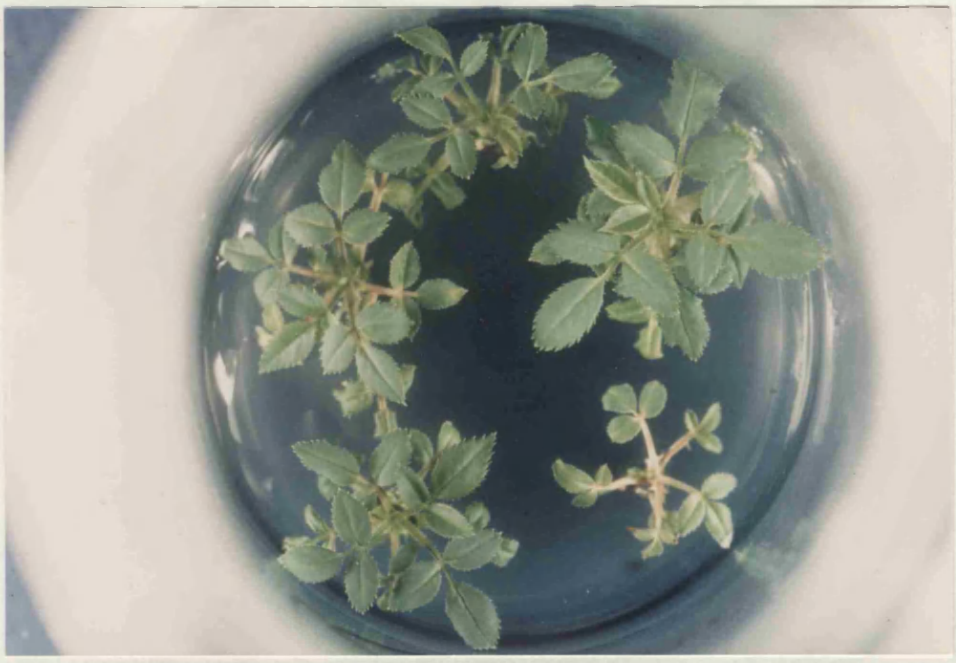
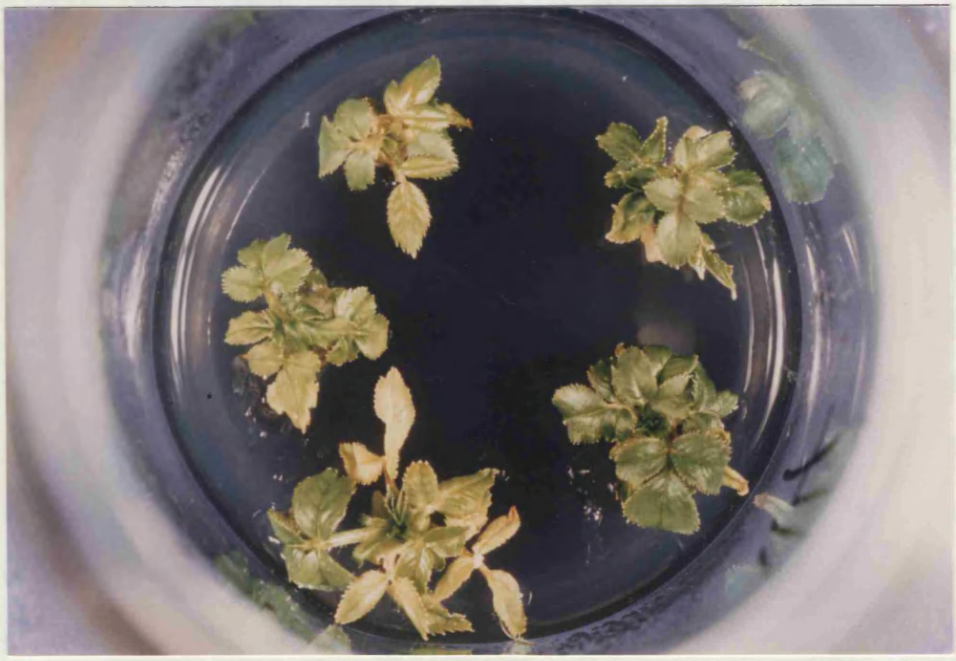


Plate 2. The growth of rose shoots cv. Peace on (a) shoot proliferation and (b) shoot elongation media, after a 4 week and 2 week culture period respectively, both containing sucrose at 30 gl^{-1}. (Scale X 2.0)

(a) Shoot proliferation

(b) Shoot elongation



8.4.2 In vivo rooting

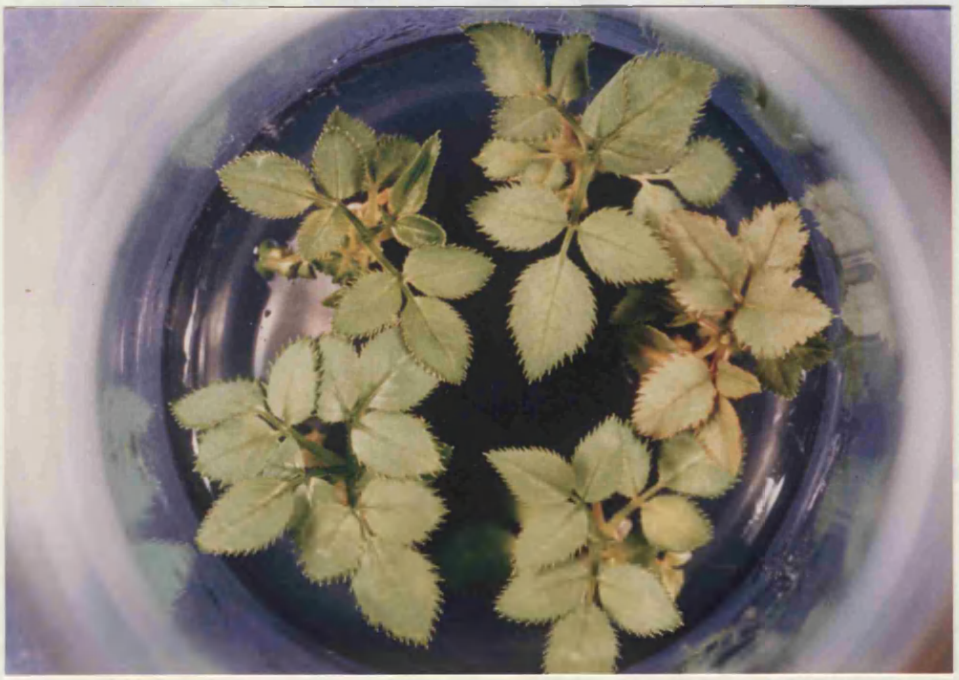
Varying sucrose concentrations during shoot elongation

As with in vitro rooting, the use of an elongation medium improved the growth and vigour of rose shoots cv. Fragrant Cloud, with the development of enlarged, healthy foliage (Plate 3a) possessing increased photosynthetic rates (Table 22). The inclusion of various concentrations of sucrose into this elongation medium had no significant effect on the physiological development of either in vitro or in vivo foliage, although these factors did vary with time under in vivo conditions. Whilst the in vitro foliage did persist after transfer to soil, there was no evidence of further growth or leaf expansion, most in vitro leaves having senesced and perished within 56d.

On transfer to soil, the in vitro foliage showed a significant improvement in CO_2 uptake after 7d, the rate decreasing over time with the onset of leaf senescence. The chlorophyll content and a:b ratio of these in vitro shoots correspondingly decreased over time as the foliage degenerated (Tables 22 and 23). The foliage developed under in vivo conditions showed increased photosynthetic rates and increased chlorophyll contents compared with in vitro foliage, irrespective of the concentration of sucrose used for shoot elongation. They showed good leaf-expansion, often being quite dark green with a bluish-grey bloom and marked marginal serrations to the leaves

Plate 3. The growth and development of rose shoots cv. Fragrant Cloud (a) cultured on a 30 gl^{-1} sucrose charcoal elongation medium (X2.0) and after (b) 7d, (c) 14d and (d) 28d following transfer to soil (X0.5).

(a) Shoot elongation



(b) After 7d in soil

(c) After 14d in soil

(d) After 28d in soil

(Plate 3, a-d). The photosynthetic rate of *in vivo* leaves showed an immediate significant increase compared with *in vitro* levels, whereas chlorophyll contents were initially similar to those of *in vitro* leaves, until the latter had senesced and their chlorophyll levels declined (Figs. 18, 19 and 20).

Table 22. The photosynthetic development of *in vitro* leaves of rose cv. Fragrant Cloud prior to and after transplanting to soil, following shoot elongation on medium containing 10, 20 or 30 gl^{-1} sucrose. (n=6, except at 56d where n=2, the data for 56d therefore being excluded from the statistical analysis)

		Stage					
		in vitro		days following transfer to soil			
µmol CO_2 h^{-1}		Shoot mult.	Elong-ation	7	14	28	56	Mean (n=24)
	10	/	43.57	71.91	65.64	52.76	20.63	58.47
g^{-1} FW	20	/	41.29	66.65	65.50	44.82	24.27	54.56
	30	20.71	55.29	66.66	72.01	52.12	49.41	61.52 NSD
Mean(n=18)		/	46.72	68.41	67.72	49.90	31.44	5%LSD= 13.66
	10	/	1.206	1.335	0.954	0.621	0.231	1.029
cm^{-2} LA	20	/	0.965	1.146	1.013	0.537	0.308	0.915
	30	0.909	1.098	1.222	0.991	0.645	0.630	0.989 NSD
Mean		/	1.090	1.235	0.986	0.601	0.390	5%LSD= 0.251
	10	/	37.26	43.55	33.20	22.33	9.41	34.08
mg^{-1} chl	20	/	28.14	39.79	34.12	19.23	16.80	30.32
	30	18.43	35.56	40.25	33.80	27.66	34.67	34.32 NSD
Mean		/	33.65	41.20	33.71	23.07	20.29	5%LSD= 7.05

Table 23. The response of chlorophyll content and a:b ratio of *in vitro* leaves of rose cv. Fragrant Cloud prior to and after transplanting to soil, following shoot elongation on medium containing 10, 20 or 30 gl^{-1} sucrose. (n=6, except at 56d where n=2, the data for 56d therefore being excluded from the statistical analysis)

		Stage						
		in vitro		days following transfer to soil				
		Shoot mult.	Elong-ation	7	14	28	56	Mean (n=24)
mg chl g^{-1} FW	10	/	1.218	1.665	1.942	2.418	1.327	1.811
	20	/	1.523	1.754	1.894	2.292	0.783	1.866
	30	1.142	1.532	1.635	2.102	1.923	1.321	1.798 NSD
Mean(n=18)		/	1.425	1.685	1.979	2.211	1.144	5%LSD= 0.229
mg chl cm^{-2} LA	10	/	0.0328	0.0310	0.0276	0.0281	0.0158	0.0299
	20	/	0.0347	0.0287	0.0291	0.0269	0.0099	0.0298
	30	0.0503	0.0309	0.0295	0.0285	0.0237	0.0166	0.0281 NSD
Mean		/	0.0328	0.0297	0.0284	0.0262	0.0141	5%LSD= 0.0034
a:b	10	/	3.29	3.03	2.97	3.22	2.18	3.13
	20	/	3.28	3.25	2.97	3.54	2.21	3.26
	30	2.64	3.39	3.14	3.10	3.19	2.75	3.20 NSD
Mean		/	3.32	3.14	3.01	3.32	2.38	5%LSD= 0.14

Fig. 18. A comparison of the photosynthetic ability of in vitro-derived (●) and in vivo-developed (o) foliage of rose shoots cv. Fragrant Cloud transferred to soil for in vivo rooting, following shoot elongation on 10 (a and d), 20 (b and e), and 30 (c and f) gl^{-1} sucrose. In vitro and in vivo data compared using t-test, probability levels given. (n=6, except at 56d in vitro leaves where n=2) M= multiplication, E= elongation.

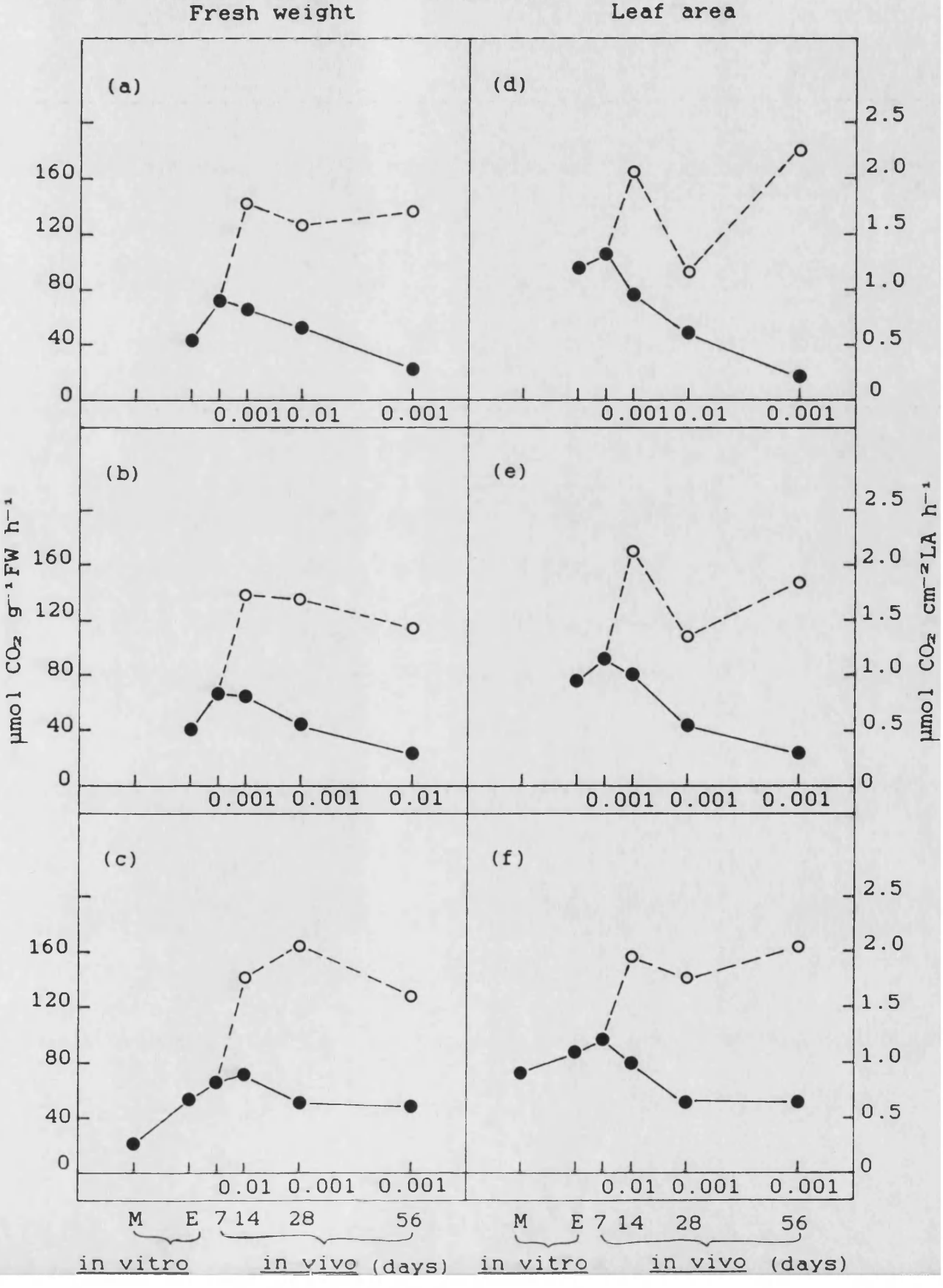


Fig. 19. A comparison of the chlorophyll content of in vitro-derived (●) and in vivo-developed (o) foliage of rose shoots cv. Fragrant Cloud transferred to soil for in vivo rooting, following shoot elongation on 10 (a and d), 20 (b and e), and 30 (c and f) gl^{-1} sucrose. In vitro and in vivo data compared using t-test, probability levels given. (n=6, except at 56d in vitro leaves where n=2) M= multiplication, E= elongation.

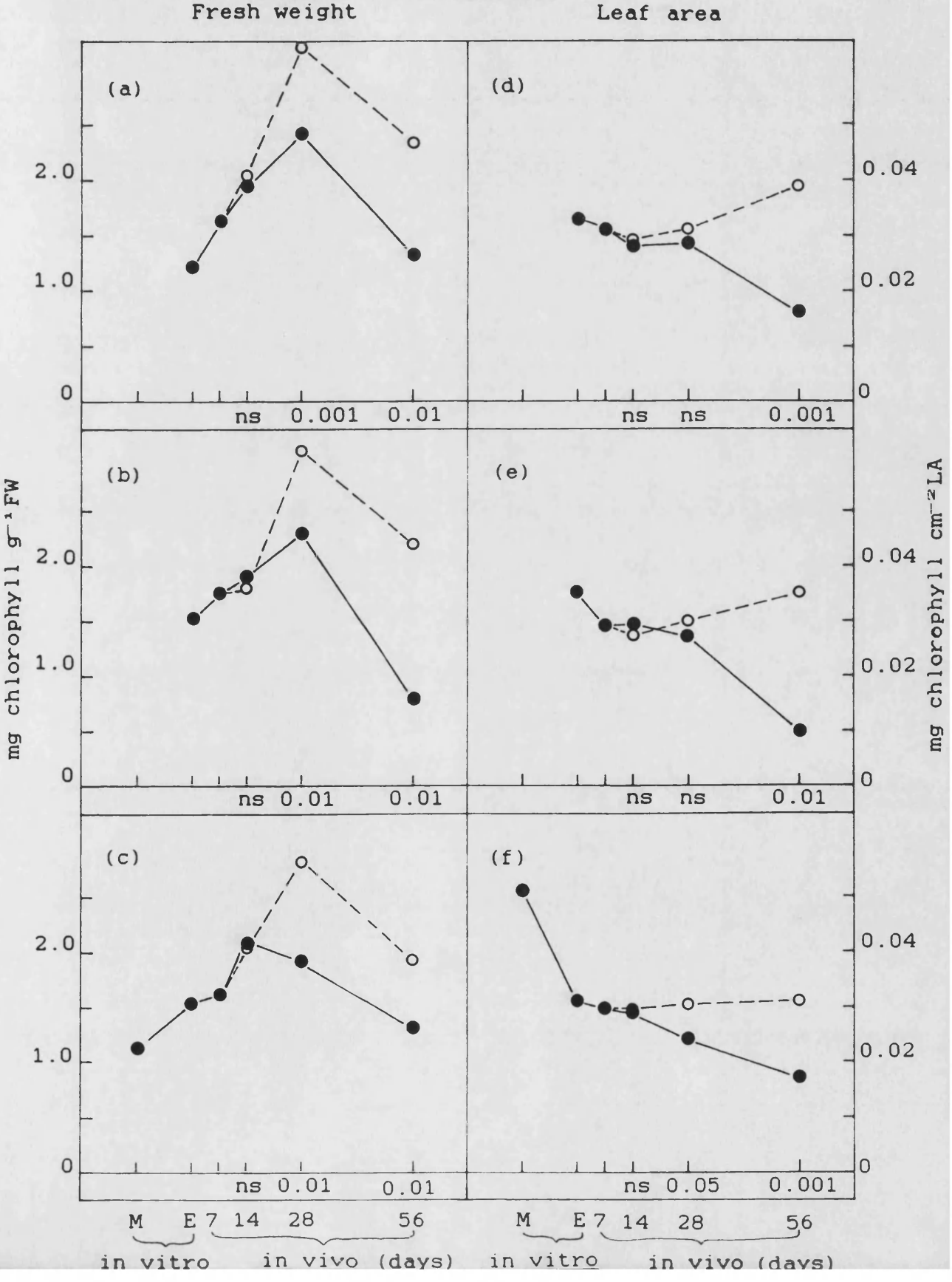
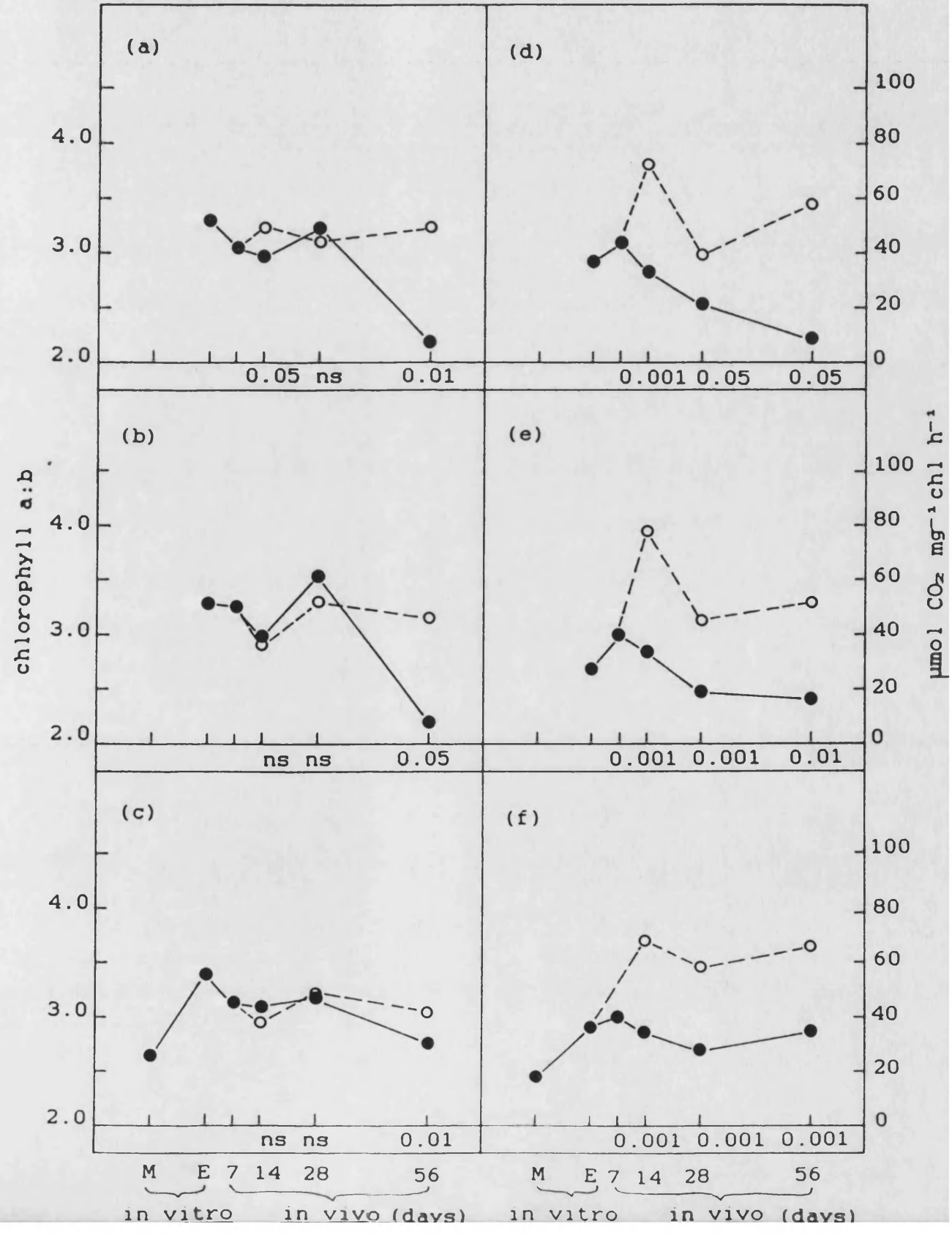


Fig. 20. A comparison of the chlorophyll a:b ratio and CO_2 uptake per unit chlorophyll of *in vitro*-derived (●) and *in vivo*-developed (○) foliage of rose shoots cv. Fragrant Cloud transferred to soil for *in vivo* rooting, following shoot elongation on 10 (a and d), 20 (b and e), and 30 (c and f) gl^{-1} sucrose. *In vitro* and *in vivo* data compared using t-test, probability levels given. (n=6, except at 56d *in vitro* leaves where n=2) M= multiplication, E= elongation.



The concentration of sucrose used for shoot elongation had no significant effect on the photosynthetic development of foliage *in vivo*, although leaves from shoots cultured at 10 gl^{-1} sucrose had significantly increased chlorophyll contents compared with those from both 20 or 30 gl^{-1} sucrose. Other than this, the concentration of sucrose played no apparent part in the physiological development of foliage *in vivo*, and the statistical analysis is not therefore presented.

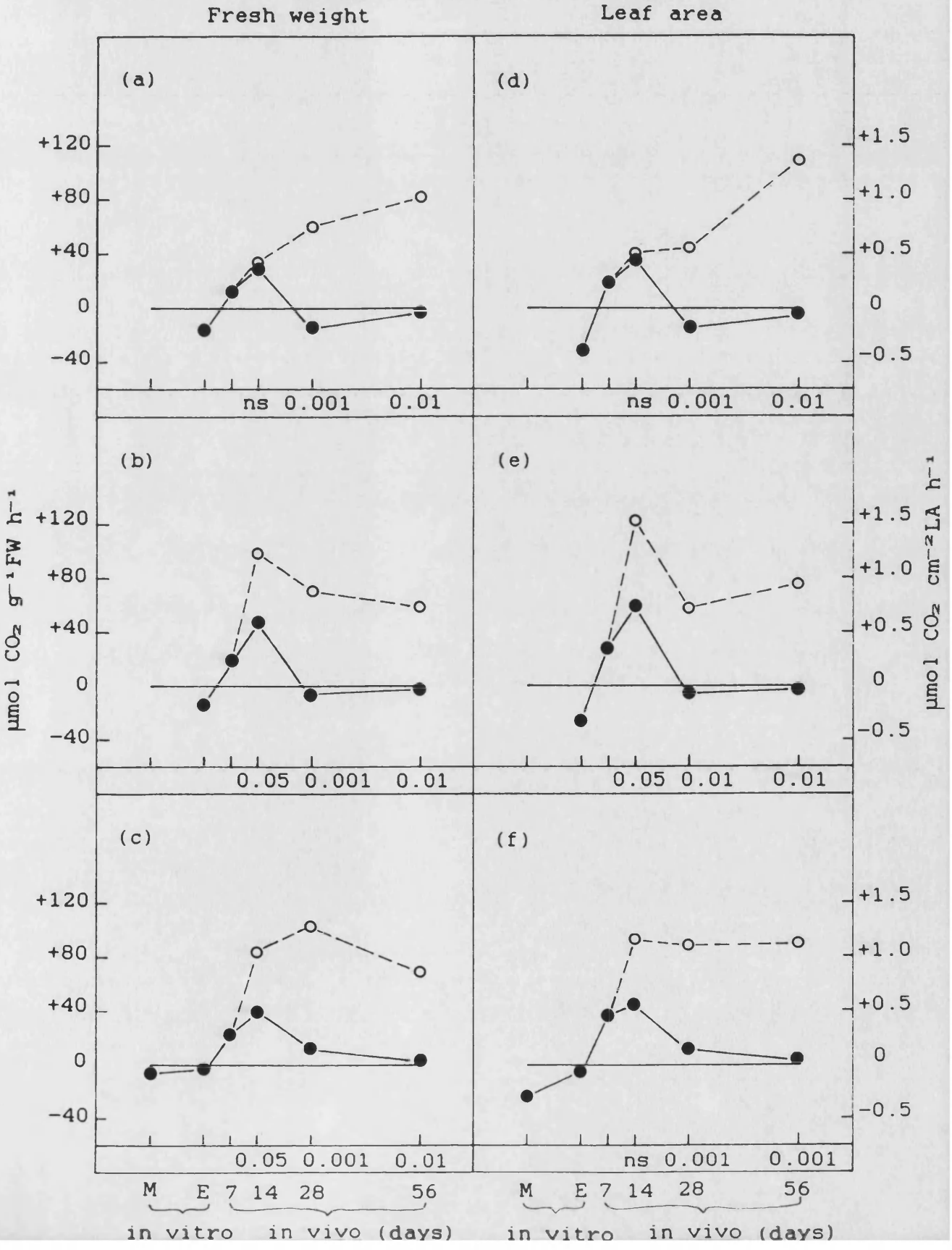
The level of photosynthetic competence shown by *in vitro* leaves was found to vary with their time in soil (Table 24). At all concentrations of sucrose, the *in vitro* foliage showed a significant increase, giving a positive carbon balance after 7 and 14d in soil, with leaves from shoots elongated on 30 gl^{-1} sucrose having maximal levels. As the *in vitro* foliage senesced however, the carbon balance correspondingly decreased to negative values. This was with the exception of leaves from 30 gl^{-1} sucrose, their carbon balance being maintained at a very low positive level, even after 28d (Table 24).

The level of photosynthetic competence shown by *in vivo*-developed foliage was positive, with a net uptake of CO_2 compared with *in vitro* foliage (Fig. 21). This was again unaffected by both the concentration of sucrose used for shoot elongation and the time in soil, and the statistical analysis is again not therefore presented.

Table 24. The degree of photosynthetic competence (ie. + or - carbon balance) shown by in vitro leaves of rose cv. Fragrant Cloud prior to and after transfer to soil, following shoot elongation on medium containing 10, 20 or 30 gl^{-1} sucrose. (n=6, except at 56d where n=2, the data for 56d therefore being excluded from the statistical analysis)

	Stage					
	in vitro		Days following transfer to soil			
µmol CO_2 h^{-1} (+ or -)	Shoot mult.	Elong-ation	7	14	28	56	Mean
(a) g^{-1}FW							
10	/	-16.87	+12.80	+29.32	-15.18	-0.52	+2.52
20	/	-15.54	+18.60	+46.19	-7.23	-2.12	+10.50
30	-7.04	-4.41	+23.30	+39.38	+12.95	+4.17	+17.80 NSD
Mean (n=18)	/	-12.27	+18.23	+38.30	-3.15	+0.51	5%LSD= 15.05
(b) cm^{-2}LA							
10	/	-0.436	+0.243	+0.444	-0.177	-0.056	+0.018
20	/	-0.319	+0.331	+0.718	-0.074	+0.025	+0.164
30	-0.310	-0.082	+0.442	+0.554	+0.159	+0.054	+0.268 NSD
Mean	/	-0.279	+0.339	+0.572	-0.031	-0.009	5%LSD= 0.259

Fig. 21. A comparison of the degree of photosynthetic competence of <u>in vitro</u>-derived (●) and <u>in vivo</u>-developed (o) foliage of rose shoots cv. Fragrant Cloud transferred to soil for <u>in vivo</u> rooting, following shoot elongation on 10 (a and d), 20 (b and e), and 30 (c and f) gl^{-1} sucrose. <u>In vitro</u> and <u>in vivo</u> data compared using t-test, probability levels given. (n=6, except at 56d <u>in vitro</u> leaves where n=2) M= multiplication, E= elongation.



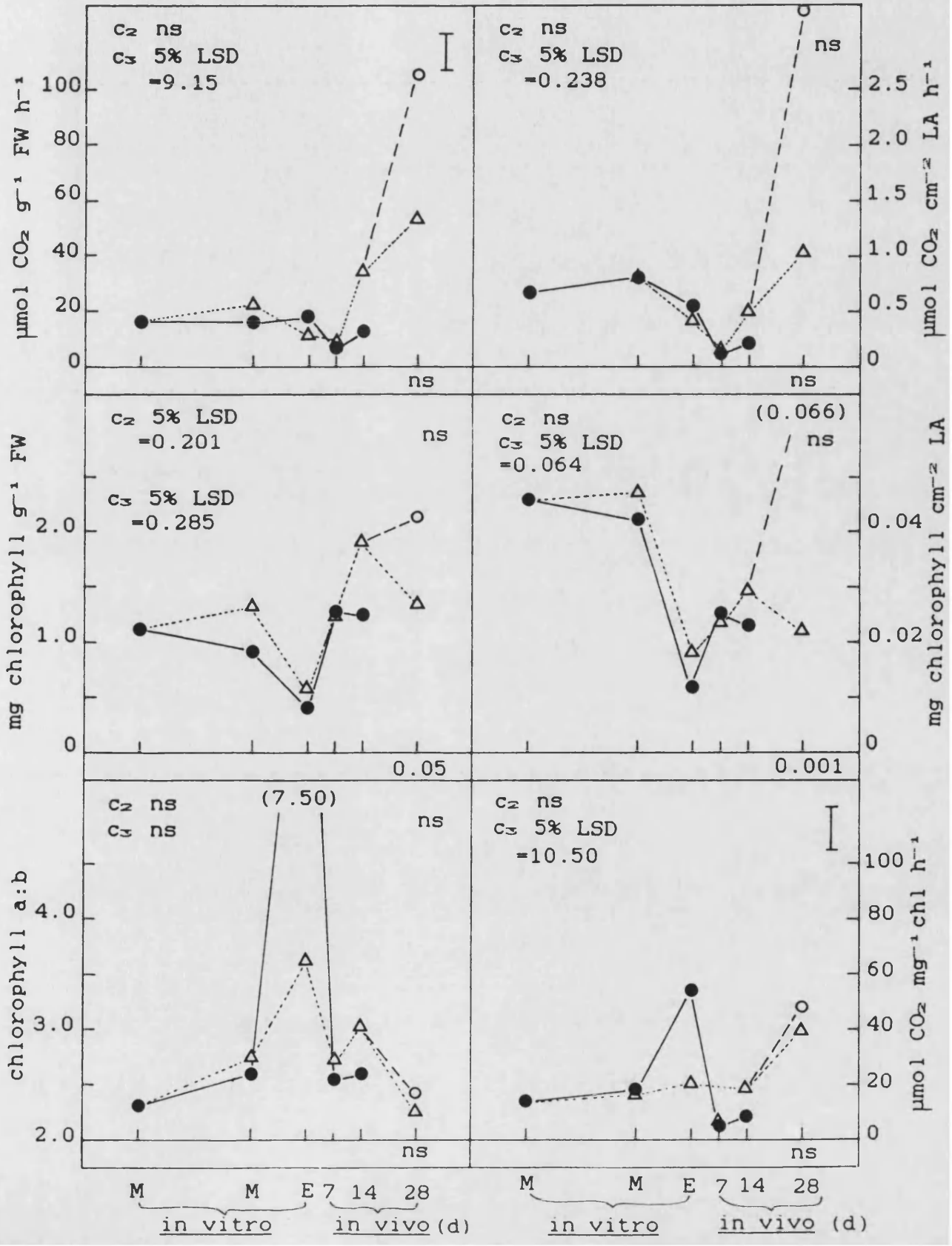
Decreasing sucrose concentrations prior to and during shoot elongation

The effect of 'constant' and 'decreasing' sucrose concentrations prior to rooting *in vivo* may significantly influence the physiological development of *in vitro* foliage. Shoots of Peace however, remained short and compact even after culture on the charcoal elongation medium. Their close proximity to the soil surface tended to encourage damping off with the result that very few shoots of Peace survived longer than 14d in soil, and the data obtained was rather variable.

The concentration of sucrose had no significant effect on the photosynthetic ability of *in vitro* leaves of Peace, although the rate of CO_2 uptake showed a large increase for the 'decreasing' sucrose treatment after 14 and 28d in soil (Fig. 22). Similarly, chlorophyll concentrations showed a large increase after 14-28d in soil, the 'decreasing' sucrose treatment also giving higher chlorophyll contents compared with the 'constant' sucrose. The chlorophyll a:b ratio showed a very variable pattern over time in soil, as indeed did all the factors assessed (Fig. 22). *In vitro* leaves from both sucrose treatments showed a negative carbon balance, those from the 'decreasing' treatment being slightly less negative than the 'constant'. This difference was not significant however (Fig. 24).

In vitro leaves of Fragrant Cloud from the 'decreasing' sucrose treatment similarly showed

Fig. 22. An assessment of the physiological development of rose shoots cv. Peace prior to and after transfer to soil, following culture on 'constant' (●) and 'decreasing' (Δ) sucrose. <u>In vitro</u> foliage (Δ) compared with <u>in vivo</u> (o) after 28d using t-test. Bar represents 5% LSD for the $c_2 * c_3$ interaction; c_2=sucrose treatment, c_3=stage in rooting procedure. (n=6, except 28d, where n=2) M= multiplication, E= elongation.



increased photosynthetic rates compared with 'constant' sucrose (Fig. 23), and a less negative carbon balance (Fig. 24). Indeed, these *in vitro* leaves showed a positive carbon balance after 14-28d, with the development of *in vivo* foliage. The chlorophyll a:b ratio was again rather variable with time in soil, decreasing to below 2.7 after 14-28d.

The *in vivo*-developed foliage showed increased rates of CO_2 uptake, increased chlorophyll contents and a positive carbon balance compared with the *in vitro* leaves, although as leaves were only assessed up to 28d, these improvements were not all significant.

Rooted weaned plantlets were transferred to individual pots after 28d and left to grow on under normal glasshouse conditions. They appeared healthy, with glossy green foliage and vigorous growth. Plants of Iceberg and Fragrant Cloud were seen to develop flower buds fairly rapidly, Peace remaining smaller with more compact sturdy growth (Plate 4).

Fig. 23. An assessment of the physiological development of rose shoots cv. Fragrant Cloud prior to and after transfer to soil, following culture on 'constant' (●) and 'decreasing' (Δ) sucrose. In vitro foliage (Δ) compared with in vivo (o) after 28d using t-test. Bar represents 5% LSD for c_2*c_3 interaction; c_2=sucrose treatment, c_3=stage in rooting procedure. (n=6, except 28d, where n=2) M= multiplication, E= elongation.

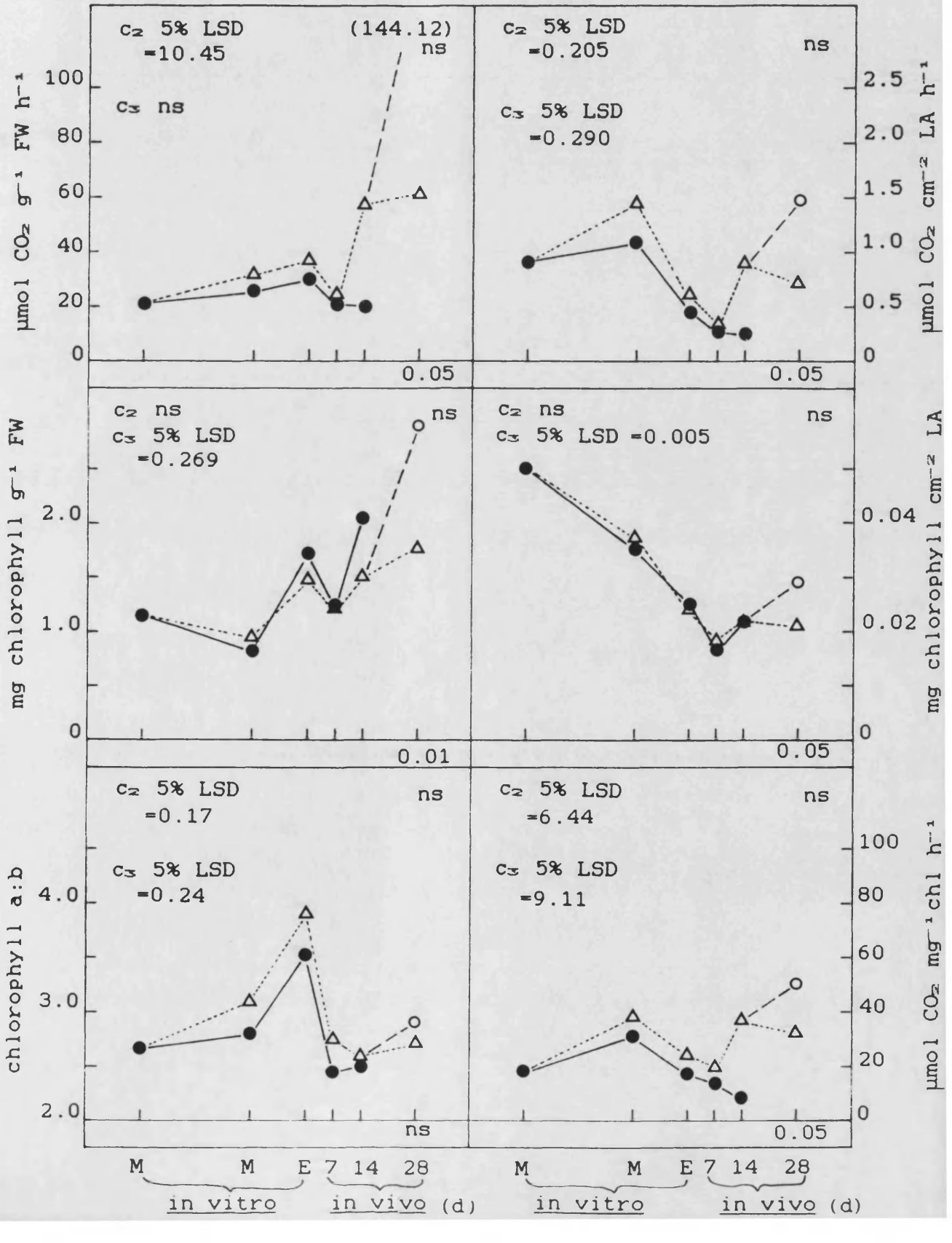


Fig. 24. An assessment of the degree of photosynthetic competence of rose shoots cvs. Peace and Fragrant Cloud prior to and after transfer to soil, following culture on 'constant' (●) and 'decreasing' (Δ) sucrose. In vitro foliage (Δ) compared with in vivo (o) after 28d using t-test. Bar represents 5% LSD for the $c_2 * c_3$ interaction; c_2=sucrose treatment, c_3=stage in rooting procedure. (n=6, except 28d, where n=2) M= multiplication, E= elongation.

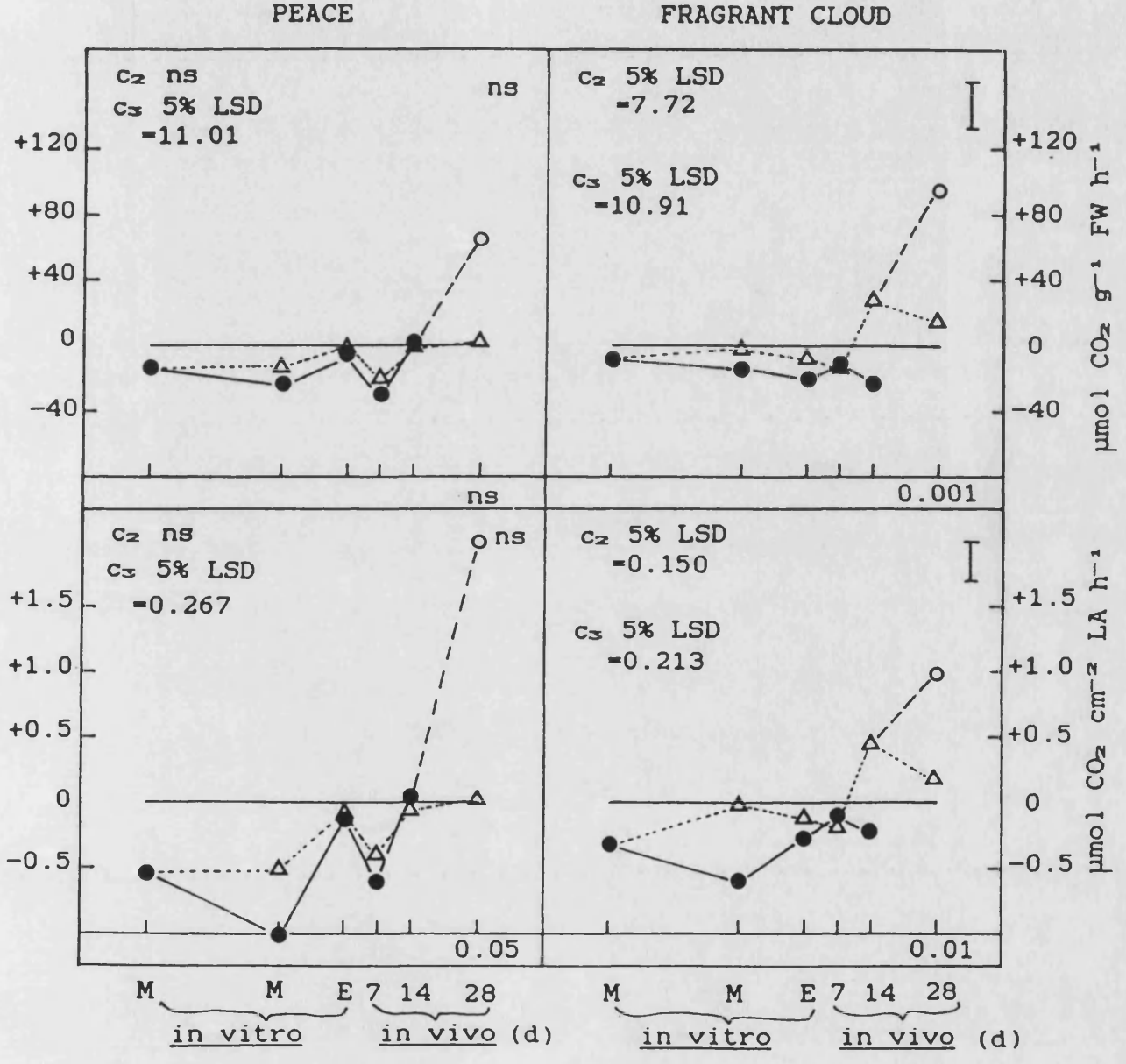


Plate 4. The growth of tissue-cultured rose plantlets cvs. Iceberg, Peace and Fragrant Cloud after 112 (Iceberg and Peace) and 56d in soil, following transfer to individual pots. (Scale X0.2)

8.5 DISCUSSION

The results presented in Chapter 3, show that manipulating the concentration of sucrose supplied in the culture medium can be used to enhance the photosynthetic ability of rose shoots *in vitro*. The influence of sucrose concentration on the rooting and establishment of tissue-cultured plantlets is less clear, and indeed its success may depend primarily on the state of the propagule ie. on a good healthy, vigorous plantlet, its photosynthetic status being of secondary importance if the shoot is in poor condition. If however, photoautotrophic shoot growth can be attained *in vitro* prior to transfer to soil, establishment can be significantly improved, with no lag phase in the development of new growth. Such leaves retained from culture do not degenerate, but persist and contribute significantly to the growth of the establishing plantlet (Grout and Donkin, 1985; Short *et al*, 1985; Grout *et al*, 1986). Previous work with *Rosa* was unsuccessful in achieving photoautotrophic growth *in vitro*, and therefore this aspect of rose shoot culture and its influence on rooting cannot be studied.

Whilst the majority of studies with rose involve *in vitro* rooting, this technique did not prove successful in this study. This was despite the use of a charcoal elongation medium prior to rooting, which produced healthy vigorous shoots with large, well-expanded leaves. On transfer to auxin-medium, shoot vigour rapidly declined with a loss of chlorophyll and leaf senescence.

Short *et al* (1981) also working with rose, report root initiation to occur within 21d on a reduced mineral salt medium (with NAA), but that maintenance of shoots on cytokinin-free medium for longer than 21d results in the onset of leaf senescence. In this study, rose shoots were cultured on elongation medium for 14d and then on rooting medium for another 14d; thus the observed decline in shoot vigour could be due to their being without cytokinin for longer than the 21d period suggested by Short *et al* (1981). The loss of chlorophyll may be explained by the suggestion that cytokinins are involved in the differentiation and development of chloroplasts and chlorophyll (Parthier, 1979; Nowak *et al*, 1986), its absence resulting in chlorophyll breakdown.

In vitro formed roots possessed an abnormal structure compared with those developed in soil, being thick, unbranched and lacking in root hairs. When transferred to soil, these roots degenerated within 14d, successful establishment thus depending on the growth of roots initiated under soil conditions. This suggests little advantage in the formation of roots *in vitro* prior to soil establishment, the removal of this stage reducing both the length and cost of the weaning process. For rose at least, the direct transfer of unrooted cultured shoots to soil, and their treatment with an auxin-powder dip gave successful rooting and establishment, with high rates of survival and good quality plantlets.

The majority of *in vitro* rooting studies use a solid

medium, although a few reports also investigate the use of liquid media. Grout and Aston (1977, 1978) report Brassica plantlets rooted in shaked liquid culture, to have little epicuticular wax and poor vascular connection between root and shoot. Similar plantlets rooted on solid medium had increased levels of wax (although still far less than seedling material) and were better able to control water loss. A study on the rooting of Liquidambar styraciflua by Lee et al (1986) however, indicates superior rooting with the use of a liquid rather than a solid medium. This was reflected by a greater percentage of rooted shoots, an improved rate of rooting, more roots per plant and increased development of root hairs.

Other studies with Brassica and Chrysanthemum report high growth rates and the development of extensive root systems using sorbarods soaked with liquid rooting medium (Short et al, 1985; Short and Roberts, 1986). These roots appeared to be functional, as rapid growth continued following transfer of the plantlet plus sorbarod to soil. The use of sorbarods for the rooting of rose shoots proved to be very unsuccessful, with rapid loss of vigour, although may also be due to the fact that the developing roots did not grow down into the sorbarod, but went out along its surface. The two species used by Short et al (1985) ie. Brassica and Chrysanthemum both show vigorous growth in vitro compared with the slower, more compact growth of Rosa, and it may be this feature which enables them to continue healthy growth in culture with

the use of sorbarods.

The major factor limiting the potential use of sorbarods for the rooting of rose shoots would appear to be the problem of excess waterlogging. This may account for the observed horizontal growth of roots and the decrease in shoot vigour. In a study by Malim (1987) this problem was overcome by adding a smaller volume of liquid medium, together with using a less dense type of sorbarod. There were also differences between the species studied however, in their ability to root successfully in sorbarods. Two species of Ficus rooted very well using such a system, whereas shoots of Betula showed a rapid loss of vigour, with a high mortality rate. As suggested previously, the vigorous growth of Ficus compared with the slower, sturdier growth of Betula may enable shoots to grow away from the surface of the sorbarod and thus develop healthy foliage and a good root system.

There is no apparent reason why rose shoots should not root successfully in vitro, and indeed the majority of reports do use an in vitro system. There was insufficient time, during the course of this study, to develop an effective in vitro system with optimal conditions, and therefore an in vivo system was devised that was usable immediately.

There are several studies reported in the literature which follow the growth and development of plantlets after transfer to soil. The foliage persistent from

culture shows little or no change in either its anatomical or physiological characteristics, any increase in leaf size resulting from an increase in cell size rather than cell number (Donnelly *et al*, 1985; Fabbri *et al*, 1986). The *in vitro* leaves make little if any contribution to the photosynthetic status of the establishing plantlet (less than 10%), soon degenerating as new foliage is produced (Grout and Aston, 1978; Donnelly and Vidaver, 1984b). As leaves are developed within the soil environment, so their anatomy and photosynthetic ability undergoes the transition from the levels found *in vitro* to those characteristic of *in vivo* material (Donnelly *et al*, 1984, Donnelly and Vidaver, 1984a,b). After 35d in soil, the photosynthetic activity of *in vivo* leaves of *Rubus idaeus* L. can range from 165% to 200% of that found in *in vitro* material (Donnelly *et al*, 1984).

This study with *Rosa* gave similar results, the *in vivo*-developed foliage having significantly increased photosynthetic rates, and a positive carbon balance. Unlike other studies however, the *in vitro* leaves were also observed to develop a positive carbon balance, albeit at a rather low level. Both the photosynthetic rate of *in vitro* leaves and their carbon balance reached maximal rates after 7-14d in soil, this time corresponding with the emergence of *in vivo* leaves. This suggests that as the *in vivo* foliage develops, so that persistent from culture starts to degenerate, supporting

the idea of a 'storage organ' or 'cotyledonary' function for the *in vitro* leaves (Grout and Millam, 1985). Minerals contained within the *in vitro* foliage have been shown to undergo redistribution into those developing in soil, so aiding the establishment process (Wardle *et al*, 1983). It may be important therefore, to optimise the condition of the micropropagated plantlet prior to transfer to soil, so as to obtain a healthy, vigorous propagule which will root and grow on to produce a high quality plantlet. The use of a pre-transfer charcoal elongation medium, as used in this study, may be useful in achieving this (Messeguer and Mele, 1986).

The influence of media sucrose concentrations on the subsequent *in vivo* rooting and establishment of rose shoot cultures remains unclear, as no differences between sucrose treatments were seen during this study. Several studies however, suggest that sucrose may have an effect on root initiation and growth *in vitro* (Pierik *et al*, 1975), although no reports have been found concerning the interaction of sucrose concentrations and subsequent *in vivo* rooting and establishment. High sucrose concentrations may improve *in vitro* rooting and plantlet quality (Maene and Debergh, 1985), 80 gl^{-1} giving optimal rooting and dry weight for shoots of *Pinus lambertiana* (Greenwood and Berlyn, 1973). In contrast, a concentration of 12-20 gl^{-1} sucrose provides for normal root development with *Saintpaulia*, the use of lower concentrations resulting in shoot death, and higher

concentrations root inhibition (Start and Cumming, 1976). The enhancement of *in vitro* rooting by decreased concentrations of mineral salts has been suggested to be due to an increase in the ratio of sucrose:nitrogen, supporting the idea of a high sucrose requirement for rooting (Hyndeman *et al*, 1982b). At full- or twice-normal strength MS, rooting is severely inhibited, with a reduction in both the number and length of roots (Hasegawa, 1980; Hyndeman *et al*, 1982b).

The possibility of improved rooting and establishment through the use of reduced levels of carbohydrate prior to transfer to soil, is suggested by Jones (1982). His work with cultured shoots of *Dianthus* and Boston fern *in vitro* however, only indicated a marginal improvement in growth at 15 gl^{-1} sucrose compared with 30 gl^{-1}, and in opposition to this was a reduction in both the number and length of fern roots at low sucrose. Similar work with coconut finds the concentration of BAP and sucrose to be less critical in root initiation than that of NAA, although further root development may be favoured by a reduction in the concentration of auxin, sucrose and/or mineral salts (Eeuwens and Blake, 1977).

The culture medium *in vitro* used prior to the transfer of shoots to soil is likely to have a significant influence on both their establishment and further growth *in vivo*. Wainwright and Marsh (1986) find that the concentrations of sucrose and mineral salts (MS) provided during the culture period affect both the dry

weight and node number of *in vivo* established plants of watercress. Highest mean dry weights were obtained for plantlets previously cultured on 4% sucrose (compared with 1 or 2%), whereas a 2% sucrose medium resulted in the highest mean number of nodes. The concentration of MS salts had no effect on plantlet weight, but increased concentrations caused a significant decline in the number of nodes obtained *in vivo*. A culture medium containing 4% sucrose and full strength MS gave rise to plantlets with greatest mean dry weights, suggesting an interaction between the two components. This may again be related to the ratio of sucrose:N_2, maximising the size of *in vivo* established plantlets through improved starch reserves.

The use of an elongation or pre-transplanting medium would seem to significantly improve both the vigour and successful establishment of rose shoots. A similar technique has been used, prior to the transfer of *Gladiolus* shoots to soil (Ziv, 1979). Rooting and establishment was significantly improved by culturing shoots onto a pre-transplanting medium containing low sucrose, half strength mineral salts and activated charcoal. This, in combination with the use of increased irradiance levels, acted as a hardening stage, preventing the desiccation of plant material on transfer to soil and encouraging continued growth with no dormancy period. The roots that developed under such conditions were also found to be functional.

In conclusion therefore, rose shoots may be

successfully rooted and established in soil following pre-culture on a charcoal elongation medium to promote shoot vigour and extension. Whilst there appeared to be little benefit in the use of varying sucrose concentrations during this pre-transfer period, it is not possible to draw any definite conclusions from this study alone. The interaction of sucrose and activated charcoal within the elongation medium is unknown, the latter component possibly reducing or even eliminating the effect of sucrose concentration. Only three cultivars of rose were used in this study, with only one (Fragrant Cloud) being successfully transferred to soil with an *in vivo* rooting system. There is great scope for further investigation, in particular into the stage at which the shoots are, or can be, exposed to different levels of sucrose, which might prove more influential on post-culture performance. Only one rooting system was employed in this study and others should be studied, as a different procedure may offer the possibility of improved results.

Perhaps the most important prerequisite for successful establishment is a healthy vigorous shoot, this also ensuring the production of a high quality product. In this study with rose at least, it would seem that the *in vitro* foliage may make some contribution to shoot establishment. The development of a positive carbon balance after 7-14d correlates with the appearance of leaves developed *in vivo*, the redistribution of nutrients

and minerals contained within the *in vitro* foliage possibly resulting in its senescence and degeneration soon afterwards.

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CHAPTER 9.

FINAL DISCUSSION AND CONCLUSIONS

In economic terms, if a plant species is to be successfully micropropagated, the costs of production have to compete with plants produced by traditional methods of macropropagation, or have superior characteristics which increase the value of the micropropagated plant (de Fossard and Bourne, 1977; Donnan et al, 1978). Micropropagated plantlets are genetically identical to the parent plant (allowing for somaclonal variation), which is clearly of benefit in plant breeding terms, this factor also making them superior to seed-raised plants. If however, they are too expensive to produce compared with conventional cuttings or seed-raised material, it becomes uneconomical to use the micropropagation technique eg. vegetable crops (seed-raised), and conventional methods are maintained. Thus any improvements in the production of tissue-cultured plants, in terms of increased productivity, improved quality, reduction in the cost and labour involved will be of commercial interest. Before a species is tissue-cultured therefore, it becomes necessary to balance the advantages and disadvantages of micropropagation against those for macropropagation (Dixon, 1986).

The advantages of the tissue-culture system include (1) a rapid multiplication rate so that large numbers of plants can be achieved quickly, (2) these plantlets will possess a high health status, with the possibility of virus-eradication, (3) the technique is flexible and can be adapted to suit a variety of species and (4) has also

proved effective with a variety of species. This is counterbalanced by (1) the tissue-cultured plantlet is a rather delicate product resulting in a rather prolonged establishment period, (2) the mother stock plant needs care to reduce pest and pathogen contamination and (3) trained staff and high-grade facilities are required. Many of the stages in micropropagation involve the handling of single shoots, meaning that the whole process is very labour-intensive and time-consuming.

The economic value of micropropagation to a particular species depends therefore, on both the cost of production and on the quality and hence monetary value of the product. Any improvement in the micropropagation system may prove to be of great commercial benefit, in particular at the weaning and establishment stages, during which time large numbers may be lost if conditions are not optimal.

There are numerous factors of both the _in vitro_ and _in vivo_ environments which may be altered in order to try and improve the successful tranfer of plantlets to soil, in addition to improving the quality and vigour of the propagule itself. Maene and Debergh (1985) discuss the various factors which may be altered for the optimalisation of plantlet transfer to soil, _Cordyline terminalis_ L. Kunth. being used as the test plant. None of the non-hormonal components were found to be critical for shoot elongation and subsequent rooting _in vivo_, in

contrast to the dramatic effects of cytokinins (especially BA) and auxins on proliferation and rooting respectively (Norton and Boe, 1980). The spectrum of light used for culture growth eg. warm-white, cool-white, grolux, fluora fluorescent tubes, also had no significant effect, although the irradiance of light clearly did. Low irradiance (20 $\mu mol\ m^{-2}s^{-1}$) stimulated uniform shoot elongation *in vitro*, but delayed the formation of plantlet growth *in vivo*. This is supported by other reports in the literature whereby shoots are proliferated under low light (1000lux) and rooted under high light (3000lux) (Bressan and Kim, 1980; Bressan *et al*, 1981, 1982).

As was the case with rose shoots *in vitro*, proliferation under high light results in the stunting of plantlets and leaf senescence (Bressan and Kim, 1980). The use of high light during *in vitro* rooting however, shows no such adverse effect on shoot growth, suggesting light to be acting in two different ways during the 2 stages, which correspondingly may have different light requirements. The use of high light as a tool for the improvement of rooting and establishment has been reported in many studies, including several with rose (Bressan and Kim, 1980; Bressan *et al*, 1981, 1982; see also Chapter 6). High light both prior to transfer (ie. during rooting *in vitro*) and following transfer to soil has proved greatly beneficial for shoot establishment, with increased root initiation and root number (Bressan

et al, 1982). For shoots rooted within the soil environment, the use of increased levels of irradiance for 2 or 3 weeks prior to transfer combines good shoot production with excellent root formation in vivo (Maene and Debergh, 1985).

The transfer of tissue-cultured plantlets to soil under increased levels of irradiance accelerates the 'normalisation' of leaves developed in vivo ie. the return to control-type leaves (Donnelly et al, 1984, 1985). Associated with this is an increase in leaf area and dry matter content, suggesting high light to 'boost' the photosynthetic system, as has been found with Asparagus (Hasegawa et al, 1973; Murashige, 1974). Thus the poor vigour shown by rose shoots proliferated under 'high' light (20 Wm^{-2}) in this study correlates with several other reports in the literature, and does not mean that rooting will also be adversely affected under similar conditions. Although 20 Wm^{-2} is not particularly high, it is relatively high compared with the levels usually used for plant tissue-culture ($\sim$ 10 Wm^{-2}).

Improved rates of CO_2 uptake and the possibility of photoautotrophic growth in vitro are both of commercial interest in the improvement of the weaning and establishment stages. Embryo-cultured coconut seedlings show large losses when transferred to soil, with only 20-30% survival (Malijan and Del Rosario, 1986). Similar problems have also been encountered with date palm (Twyford Plant Laboratories Ltd. - private communication)

and in both cases the limited photosynthetic ability has been suggested to be a contributing factor. Photosynthetic competence in coconut seedlings commenced 8 weeks after transfer, correlating with the full expansion of a new leaf (Malijan and Del Rosario, 1986).

The studies with rose showed sucrose to have a significant effect on the photosynthetic rate of shoots, this being of potential use in the manipulation and increase of photosynthesis in vitro. The foliage developed in vitro has a net negative carbon balance ie. is photoheterotrophic, and on transfer to soil, perishes, contributing very little to the photosynthetic state of the establishing plantlet. Whilst it has proved possible to produce photoautotrophic callus and single cell cultures in vitro, which are capable of growth independent of an organic carbon source (ie. a sugar), there are very few cases involving whole shoots. The only reports of autotrophic shoot growth in vitro involve species such as strawberry, Brassica and Chrysanthemun which show vigorous growth in culture (Grout and Donkin, 1985; Grout et al, 1986; Short and Roberts, 1986). This may be one of the factors enabling such species to show sucrose-independent growth, the comparatively slow, sturdy growth of rose found in this study preventing shoot growth in the absence of a sugar. Even attempts to wean rose shoots onto progressively lower concentrations of sucrose proved unsuccessful. Photoautotrophic shoots possess a positive carbon balance, enabling the

transferred plantlet to show continued growth immediately after transfer to soil. This is clearly of great commercial benefit, reducing the length of the weaning process and resulting in improved establishment.

Whilst both complete sucrose removal from the medium and a gradual decrease in sucrose concentration proved unsuccessful in inducing photoautotrophic growth for rose, Grout et al (1986) report photoautotrophic strawberry growth in vitro following complete defoliation of shoot cultures, leaving only the petioles remaining. Such shoots, on transfer to a sucrose-free medium, appeared able to use the stored carbohydrate to support the production of new foliage, with a net positive carbon balance and a high RuBP carboxylase activity. This compares with earlier studies with Brassica, where photoheterotrophic shoot cultures possessed a net negative carbon balance and lower levels of RuBP carboxylase activity compared with control material (Grout and Donkin, 1985). The reduction in the activity of the enzyme responsible for CO_2 fixation would appear to be limiting any further increase in CO_2 uptake or the development of photosynthetic competence.

A similar approach with rose ie. defoliation of shoots, again proved unsuccessful (no data presented), shoots perishing within 4-8 weeks of transfer to a sucrose-free medium. As suggested previously, the difference in growth between rose and strawberry shoots in culture, the latter species having much more vigorous,

prolific growth, may enable strawberry to adapt to a sucrose-free medium and correspondingly develop photosynthetically competent foliage, whereas rose is unable to.

These photoautotrophic shoot cultures however, are reported to have greatly decreased rates of growth, this clearly being an undesirable factor associated with sucrose-independent growth. It would seem necessary therefore, to try and enhance shoot growth, possibly by altering some other factor of the in vitro environment eg. gaseous environment, light etc. in conjunction with the sucrose component. The possibility of increased light levels and sucrose decrease/removal just prior to transfer (ie. during in vitro rooting) may prove to be beneficial, increased light being widely reported to enhance rooting and transfer of shoots (see Chapter 6).

The gaseous environment developed within the culture vessel may significantly affect the growth and greening of cultures. The accumulation of ethylene inhibits the greening of spinach cell cultures (Dalton and Street, 1976), whilst high concentrations of CO_2 are reported to reduce the growth rate of Cinchona shoots in vitro (Hunter et al, 1986). Studies with Magnolia in vitro report CO_2 concentrations as high as 14% being detected, with ethylene concentrations increasing to as much as 2-3ppm after 9 weeks (De Proft et al, 1985). The use of alternative vessel closures has therefore been considered, to enable better gaseous exchange between the

culture vessel and the outside environment. Whilst a bung type closure led to CO_2 accumulating up to 15%, a foil plus cling film closure only led to a 0.7% accumulation (Hunter et al, 1986), and associated with this decrease was a doubling in shoot dry weight and an increased photosynthetic activity. Better gas exchange can thus prevent the accumulation of such gases, improving shoot growth and greening of cells, as well as reducing the high levels of relative humidity found in culture vessels (Maene and Debergh, 1985).

The majority, if not all reports of photoautotrophic cell and callus growth involve the removal of sucrose from the medium, either in one step or over several successive subcultures, in conjunction with CO_2 enrichment (to 1 or 2%) and an increase in light levels (LaRosa et al, 1984; Chaumont and Gudin, 1985; Tyler et al, 1986). The increase in CO_2 concentration presumably compensates for the reduction or removal of media sucrose, the increased irradiance 'boosting' the photosynthetic system. Whilst the manipulation of these 3 factors is clearly of use for cell and callus cultures, the effect on whole shoots is unknown and requires a full study and assessment. The adverse effect of high light on proliferating shoot cultures may limit the use of light during this stage, although the removal of sucrose and increase in CO_2 and light just prior to transfer may prove effective.

The interaction of CO_2 concentrations and light has

been studied *in vivo* with seedling material of *Liquidambar styraciflua* (sweetgum) and *Pinus taeda* (loblolly pine) (Tolley and Strain, 1984). An increase in the concentration of atmospheric CO_2 led to a significant increase in height, leaf area, basal stem diameter and total dry weight of sweetgum seedlings grown under high light (1000 $\mu mol\ m^{-2}s^{-1}$), the increase in dry weight being associated with a CO_2 enhancement of net assimilation rate. The reduction in seedling growth under low light (250 $\mu mol\ m^{-2}s^{-1}$) could be compensated for by an increase in CO_2 concentration, although seedlings of loblolly pine did not show a similar response, contradicting the sweetgum data to some extent.

Elevated CO_2 concentrations within *in vitro* systems have been shown to promote the growth of cell suspensions of *Catharanthus roseus*, the rate of CO_2 fixation being dependent on the partial pressure of CO_2 in the culture environment (Maurel and Pareilleux, 1986). This has been studied further with a view to stimulating the growth of *in vitro* propagated grape vines following transfer to soil (Lakso *et al*, 1986). The growth of shoots appeared to be limited by the supply of photosynthetic carbon, supported by the observation that CO_2 enrichment led to a 2 and 4 fold increase in dry weight after 20 and 30d in soil respectively, compared with controls. Root growth was especially improved, being ~ 6 times greater than controls by 30d.

Thus both *in vivo* and *in vitro* studies indicate that

manipulating the concentration of CO_2 may lead to a significant improvement in the growth of both whole shoots and seedlings, an increase in irradiance also proving to be beneficial. There are very few studies reported in the literature concerning this area of plant tissue culture, and much more work is required as significant improvements would appear to be attainable.

The use of a gibberellin or chilling treatment has been shown to improve the establishment of *in vitro* propagated plum cv. Pixy. Chilling for 2 months at 0°C before potting on (following transfer to soil) or post-potting sprays of 200ppm GA_3 were both effective in improving growth. Such plants went on to produce fewer multiple stems and when treated in early spring could be transferred to the field in mid-summer (Howard and Oehl, 1981).

The optimalisation of the transfer of tissue-cultured plantlets is reviewed in more detail by Maene and Debergh (1985). As well as the factors mentioned previously, the addition of liquid media supplements to established cultures is discussed as an alternative to transferring to fresh medium. This could be of use at the end of the multiplication stage for the induction of shoot elongation, and following elongation for root induction. This technique could help reduce manipulation costs and also improve the production of shoots through a reduction in stress, as the cultures no longer have to be

physically cut.

There are numerous other factors of the *in vitro* environment which can be studied and used to improve the proliferation, weaning and establishment of cultured shoots. Such studies may particularly improve those species which still prove difficult to establish in culture. Cohen (1986) reports explant source to be a key factor in success, involving the pre-culture environment of the mother plant, explant position etc. Similarly, there may be seasonal variation between shoot and root formation depending at which time of year explants are taken and introduced into culture (Norton and Norton, 1986a). Shoot proliferation may be influenced by the consistency of the medium and the density of shoots within a culture vessel (Chun *et al*, 1986). The number of *Populus* shoots per explant is reported to be increased on a liquid medium, whilst increasing the number of shoots per vessel slowed both growth and shoot production. This is not unexpected as fewer nutrients and minerals will be available to each shoot, thus reducing the rate of growth.

Maintaining proliferating shoots in culture for extended periods of time may lead to changes in productivity, proliferation rates declining after several generations (Norton and Norton, 1986b). Associated with this was an decrease in shoot length, leaf size and an increase in callus formation. This apparently irreversible decline may be due to genetic or

epigenetic changes resulting from fluxes in cytokinin, nutrient status or the elimination of seasonal fluctuations. The problems incurred with rose cv. Iceberg mentioned in Chapter 8 could be due to the length of time maintained in culture, Iceberg perhaps being more susceptible to these changes than cv. Peace. Explants taken from parent plants during the autumn period were also much slower to grow on, the seasonal environment possibly influencing their growth in culture.

Other more obvious factors such as plant growth regulators (Norton and Boe, 1980), medium pH (Martin and Rose, 1976; Wolfe et al, 1986), alternative sugars etc. influence shoot growth in culture, and may be manipulated so as to obtain optimal conditions for shoot proliferation in vitro. Any increase in shoot production and quality will be of great commercial importance, reducing the cost of each plantlet sold and making the whole process of micropropagation more economical and competitive with standard methods of macropropagation.

Thus whilst it did not prove possible to develop photoautotrophic cultures of Rosa, the results presented in this thesis may help to identify factors which influence shoot growth and physiological development in vitro, showing the way for further work. There is great scope for improved photosynthetic abilities and encouraging photoautotrophic growth within the culture environment, the importance of this for improved plant tissue culture being, at present, greatly underestimated.

I would like to end this thesis by quoting Grout and Aston (1978b), their ideas and suggestions still holding true today, nearly 10 years later:

"If methods of meristem culture are to achieve their potential for a wide range of species, hardening programmes must be designed to ensure survival of all the regenerates. The photosynthetic status of the cultures must be established and attempts made, by modifying the medium or the culture environment, to induce full autotrophic capacity as early as possible after transplanting, or even before it. The value to breeding, propagation and conservation of such a system is limited if survival of all the regenerates cannot be guaranteed".

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Appendix

The commercial production of roses, September 1986.

MICROPROP ROSES

MICROPROPAGATION SERVICES (E.M.) LTD.
BROOKSIDE NURSERIES
KIRK LEY ROAD, EAST LEAKE
LOUGHBOROUGH, LEICS. LE12 6DE
Telephone: East Leake (0509 82) 6295

ROSE VARIETIES - SPRING 1986

X denotes varieties out of stock

Floribunda

Allgold	Bright Yellow	
Anna Ford	Deep Orange	8
Arthur Bell	Br. Yellow	
Beautiful Britain*	Orange	8
X Bonfire Night*	Orange Scarlet	9
Chinatown	Br. Yellow	
Dearest	Rosy Salmon	
Dorothy Wheatcroft	Orange Red	
X Eliz. of Glammis	Orange Salmon	
Emmerdale	Pink	
Evelyn Fison	Bright Red	
Festival Fanfare	Verm/white strip.	9
X Fragrant Delight	Orange Salmon	7
Glenfiddich	Amber Yellow	8
Iceberg	White	
Korresia	Br. Yellow	10
X Living Fire*	Vermillion	4
Margaret Merril	White/Pink tint	8
Masquerade	Yellow turn Red	
Matangi	Or.Red/White rev	9
Memento	Salm.Red/Pink	8
Mountbatten	Yellow	8
Orange Sensation	Orange Vermillion	
X Orange Silk	Orange Vermillion	
Paddy McGredy	Carmine	
X Queen Elizabeth	Pink	
The Flower Arranger	Pearly Peach	7
X Tip Top	Rosy Salmon	
Topsi	Orange Scarlet	8

Miniature

Angela Rippon	Coral Pink	8
Baby Gold Star	Br. Yellow	
Baby Masquerade	Yellow turn Red	
Bit o' Sunshine	Light Yellow	
X Easter Morning*	Ivory	
Fire Princess	Bright Scarlet	
Gold Pin	Yellow	7
Hula Girl	Lt. Orange Salm.	8
Humble Herbert	Yellow/Pink tips	
Josephine Wheat.	Yellow	
Judy Fischer	Dark Pink	
June Time	Light Pink	
Lavender Jewel	Pink/Lavender	
X Little Flirt*	Or.Red/Or.Yell rev	
Magic Carrousel	White/Pink tips	
Mood Music	Apricot	
Mr Bluebird	Pink/Purple	
Orange Honey	Br. Orange	
Peek A Boo	Apricot Pink	9
Pour Toi*	Creamy White	
Scarlet Pimpernel	Scarlet	
X Simple Simon	Red	

Hybrid Tea

Alec's Red*	Cherry Red	8
Blessings	Coral Pink	4
Blue Moon	Lilac Pink	
Can Can	Deep Orange Red	8
Deep Secret	Red	
Duke of Windsor	Vermillion	8
Ernest H. Morse	Crimson	
Fragrant Cloud	Coral Red	
Grandpa Dickson	Lemon Yellow	
X Harry Wheatcroft	Scar striped Yell	8
Just Joey	Copper Pink	9
King's Ransom*	Yellow	
Mister Lincoln	Dark Red	
National Trust	Br. Red	9
Pascali	White	
Peace	Yellow/shade Pink	
Peaudouce	Primrose Yellow	11
Piccadilly	Scar/Yell reverse	
X Pot o' Gold	Yellow	10
X Prima Ballerina	Deep Rose Pink	
X Red Devil	Red	
Rose Gaujard	Carmine & White	
X Silver Jubilee*	Peach Pink & Cream	8
Sunblest	Yellow	8
Super Star	Rosy Vermillion	
Wendy Cussons	Rose Red	
Whisky Mac	Amber Yellow	8

Climber/Rambler

Albertine	Light Pink	
X Compassion	Pink/Apricot	8
Danse du Feu	Orange Scarlet	
Dorothy Perkins	Rose Pink	
Dublin Bay	Br. Crimson	12
Handel	Cream/Rose edge	
X Masquerade Climb.	Yellow turn Red	
Mermaid	Cream	
Paul's Scarlet	Bright Scarlet	
Pink Perpetue	Pink/Carmine reverse	
X School Girl	Apricot	
The New Dawn	Pale Pearl Pink	
Zephirine Drouhin	Deep Pink	

Ground Cover & Shrub

Canary Bird	Yellow	
Nozomi	Pink	8
Snow Carpet	White	10

Royalties marked in pence per plant
Growing Instructions available on request
* not available Spring 1986

The commercial production of miniature roses at Neo Plants Limited, Freckleton, Lancs., September 1986.

In the period January to August (inclusive) we produced and sold the following miniature roses. All are supplied as young weaned plants ready for potting. Plants are delivered in 40 or 60 cell polystyrene trays.

	QTY	COLOUR
Angela Rippon	34,300	Salmon
Sweet Honesty	8,400	Cream
Rugul	38,140	Yellow
Fire Princess	31,060	Red
Little Buckaroo	31,000	Red
Velvet Rosamini	49,480	Red
Charming Rosamini	9,100	Pink
Magic Carousel	14,180	Pink/White Bicolour
Baby Gold	12,420	Yellow
Salmon Rosamini	17,630	Salmon
Cinderella	5,620	White
June Time	31,400	Pink